BACULOVIRUS PRODUCED *PLASMODIUM FALCIPARUM* VACCINE

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FIELD OF THE INVENTION

This invention is in the field of recombinant *Plasmodium falciparum* polypeptides, vaccines, and vaccine formulations.

BACKGROUND OF THE INVENTION

The major merozoite surface protein of Plasmodium species has been shown to be a target of varying degrees of protective immunity against the asexual blood stages in rodent and human malaria. For example, vaccination of mice with purified P230, the major merozoite surface protein of the rodent malaria *Plasmodium yoelii*, has resulted in reduced parasitemias in comparison to controls upon intravenous challenge with a lethal dose of parasitized erythrocytes (Holder *et al.* 1981. Nature 294:361). Mice have also been protected against *P. yoelii* by passive transfer of a monoclonal antibody (Mab) specific for P230 (Majarian *et al.* 1984. J. Immunol. 132:3131) and against (rodent malaria) *Plasmodium chabaudi adami* challenge by passive immunization with a Mab specific for the homologous 250-kDa molecule of this plasmodium species (Lew *et al.* 1989. Proc. Natl. Acad. Sci. USA 86:3768). The ability to confer resistance to parasite challenge by passive transfer of antibodies suggests that antibody-mediated mechanisms play an important role in antigen-specific immunity to malaria.

Despite these findings, however, no commercially viable vaccine has been developed against the major merozoite surface antigen of the major human malaria pathogen, *Plasmodium falciparum*.

For example, using naturally derived materials, such as the precursor of the major merozoite surface protein (MSP) alone (gp195: 195,000-200,000 Da molecular species; merozoite surface protein-1 (MSP-1)), gp195 mixed with certain of its natural processing fragments, or a natural processing fragment by itself, partial protection against *Plasmodium falciparum* infection has been achieved by some researchers (Hall *et al.* 1984. Nature 311:379; Perrin *et al.* 1984. J. Exp. Med. 160:441; Patarroyo *et al.* 1987.

Vaccines 87 (Brown, Chanock, Lerner, ed.) Cold Spring Harbor Laboratory Press, CSH, NY. 117-124). An effective vaccine against *Plasmodium falciparum* should not convey merely partial protection, *i.e.* a partial lowering of parasitemia, since even low parasitemias of this organism cause serious illness. A commercially useful vaccine should substantially eliminate parasitemia. It is generally thought that an acceptable malaria vaccine that would reduce parasitemia to a low level and, consequently, result in a substantial reduction in morbidity and mortality.

In one instance, substantially complete protection using natural materials against *Plasmodium falciparum* challenge has been achieved in *Aotus* monkeys, in particular by the use of a mixture of gp195 and some of its natural processing fragments obtained by affinity purification using a Mab, designated Mab 5.2 (Siddiqui *et al.* 1987. Proc. Natl. Acad. Sci. USA 84:3014). In follow up experiments using Mab 5.2 affinity purified, parasite gp195, a correlation was found between protection against infection with *Plasmodium falciparum* and the ability of serum antibodies to strongly inhibit parasite growth *in vitro*. In particular, monkeys and rabbits hyperimmunized with Mab 5.2 affinity purified parasite gp195 in complete Freund's adjuvant produced antibodies that inhibited *in vitro* parasite growth (Hui *et al.* 1987. Exp. Parasitol. 64:519).

The difficulty in developing an effective naturally derived vaccine, however, has been compounded with the difficulty in developing an effective recombinant or synthetic vaccine. Recombinant or synthetic vaccines are desirable for several reasons. They have the potential to focus immune response(s) on the most effective portion of gp195, an important advantage since there may be decoy determinants in gp195 which prevent the most effective response. Also, more homogeneous preparations are possible using recombinant techniques than in preparations of naturally derived products. In addition, recombinant and synthetic based vaccines avoid the potential contamination of naturally derived gp195 with pathogens from its human source.

Although a number of investigators have designed and tested gp195-based synthetic peptides and recombinant products as vaccine antigens, no strongly protective vaccine has resulted. Thus, synthetic peptides corresponding to various segments of the N-terminal 83 kDa processing fragment of gp195 induced antibodies in rabbits which displayed only a low level of cross reactivity with asexual blood stage parasites (Cheung *et al.* 1986. Proc. Natl. Acad. Sci. USA 83:8328). One of these synthetic peptides, corresponding to a non-repetitive, conserved sequence, partially protected *Saimiri* monkeys against *Plasmodium falciparum* challenge (Cheung *et al.* 1986). In a vaccination study in *Aotus* monkeys using an 83 kDa processing fragment-based recombinant polypeptide produced in *E. coli* there was no significant difference between the course of infection of control animals and

animals immunized with the recombinant polypeptide. In addition, very low levels of antibodies cross-reactive with native gp195 by immunofluorescence were induced (Knapp *et al.* 1988. Behring Inst. Mitt. 82:349). A bacterial recombinant polypeptide based on a fusion of two conserved regions located towards the amino terminus and center of the gp195 molecule induced only low indirect fluorescent antibody (IFA) titers when used to immunize *Aotus* monkeys (Herrera *et al.* 1990. Proc. Natl. Acad. Sci. USA 87:4017) and two out of five immunized animals were partially protected.

Holder *et al.* studied two recombinant polypeptides which corresponded to portions of the 42 kDa C-terminal processing fragment of gp195 (p42) fused to trp E and beta-galactosidase carrier sequences, respectively (Holder *et al.* 1988. Parasite. Immunol. 10:607). While immunized animals produced high antibody titers against the carrier portion of the recombinant polypeptides, much lower titers were detected against the gp195 antigen. Some of the *Aotus* monkeys immunized with both of these recombinant polypeptides were partially protected against parasite challenge.

Murphy *et al.* (1990. Parasitology. 2:177-183) attempted to recombinantly produce portions of the p42 antigen of the Wellcome isolate of gp195 in insect host cells. gp195 is believed to exist in at least two allelic forms, of which the Wellcome isolate ("Wellcome allele") and the MAD isolate ("MAD allele") are representative (Tanabe *et al.* 1987. J. Mol. Biol. 195:273). While Murphy *et al.* reported producing a product which folded in a similar manner to the natural antigen, they did not report obtaining a purified polypeptide but only reported multiply banded antigens speculated to have resulted from post-translational processing or degradation. No follow-up immunogenicity or efficacy studies have been reported using any materials obtained.

A mixture of three synthetic peptides, one peptide from the 83 kDa processing fragment of gp195 and two non-gp195 malaria peptides, partially to completely protected monkeys against parasite challenge; a hybrid synthetic polymer including the sequences of the three synthetic peptides in addition to a circumsporozoite region was reported to provide a delay or suppression of parasitemias (Patarroyo *et al.* 1987. Nature 328:629; Rodriguez *et al.* 1990. Am. J. Trop. Med. Hyg. 43:339; Patarroyo *et al.* 1988. Nature 332:158). Field trials of this hybrid are under way. It is unclear whether any gp195 epitopes in the mixture or hybrid resulted in any protection. In addition there have been two reported studies which were unable to duplicate the prior results obtained using the peptide mixture (Reubush *et al.* 1990. Am. J. Trop. Med. Hyg. 43:355-366) or the hybrid peptide multimer (Herrera *et al.* 1991. Abstract in the IV International Congress on Malaria and Babesiosis). Thus, there has been no gp195-based recombinant or synthetic vaccine antigen which has been shown sufficiently effective against *Plasmodium falciparum*

challenge.

Accordingly, it is an object of the invention to provide recombinant or synthetic antigens, compositions comprising these antigens, and methods of use that are effective against *Plasmodium falciparum* challenge.

SUMMARY OF THE INVENTION

In accordance with these objectives, the present invention provides a composition comprising a malarial polypeptide expressed by an insect cell which contains a vector encoding the malarial polypeptide that is immunogenic in a mammalian host.

In another aspect, the invention provides a composition comprising a malarial polypeptide and an adjuvant.

In a further aspect, the invention provides methods of inducing an anti-plasmodium immune response in a primate. The immune response preferably substantially reduces parasitemia in a plasmodium-infected primate.

In a yet another aspect, the invention provides methods of making an anti-plasmodium immunogen.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures lA-B are schematic representations of a baculovirus p42 gene construct. In Fig. 1A the structure of baculovirus p42 consists of the *flg*5 leader sequence (flg_L, solid box) fused to the p42 coding region from amino acids Ala_{1333} to Ser_{1705}. In Fig. 1B synthetic DNA (solid boxes) encodes the *flg*5 leader and 5' region of p42 as a BamHI(B)/HindIII fragment. A synthetic PstI(P)/SalI(L) linker encodes the termination codon (solid box) at the 3' end of the construct. The HindIII/PstI fragment was derived from cloned parasite DNA.

Figures 2A-B depict silver stains (lane 1) and immunoblots (lane 2) of BVp42 (Fig. 2A) and Yp42 (Fig. 2B) electrophoresed in a 10% SDS-polyacrylamide gel. Immunoblots were reacted with rabbit anti-parasite gp195.

Figures 3A-B show immunoblots of purified parasite gp195 electrophoresed under nonreducing (Fig. 3A) or reducing (Fig. 3B) conditions and reacted with anti-gp195 Mab 5.2 (lane 1); rabbit anti-parasite gp195 (lane 2); rabbit anti-BVp42; (#131, lane 3 and #132, lane 4); rabbit anti-Yp42 (#93, lane 5 and #96, lane 6). Fig.3C shows immunoblots of BVp42 (lane 1, non-reduced; lane 2, reduced) and Yp42 (lane 3, non-reduced: lane 4,

reduced) reacted with Mab 5.2.

Figures 4A-C are graphs depicting a competition ELISA using purified, parasite gp195 coated plates and BVp42 and gp195 inhibitors.

Figure 5 is a graph showing ELISA titers against a BVp42 antigen of anti-parasite gp195 sera of congenic mice.

Figure 6 shows amino acid sequences of FUP isolate p42 (SEQ ID NO:2) and of the corresponding MAD (SEQ ID NO:3), K1 (SEQ ID NO:4) and Wellcome strains (SEQ ID NO:5). Amino acids shown in the MAD, K1 and Wellcome strains are those that are different from those of the FUP strain. Deletions are shown by a period. Also indicated are the sites for post-translational modification (conserved potential N-glycosylation sites are shown as filled diamonds and non-conserved sites by open diamonds; conserved cysteines are shown by filled circles and non-conserved by open circles) and the beginning of the putative transmembrane region is indicated by the arrow. Number of amino acids is according to Chang *et al.* 1988. Exp. Parasitol. 67:1.

Figures 7A-C show the nucleic acid sequence of the original BVp42 construct (SEQ ID NO:1) described in Example 1. The construct codes for a *flg*5 leader polypeptide adjacent to amino acids 1333 to 1705 of the FUP isolate gp195, as those amino acids are numbered in Figure 6.

Figures 8A-D show the ELISA titer of *Aotus* monkeys immunized with BVp42 adjuvant preparations. ELISAs were performed as described by Chang *et al.* 1989. The endpoint of ELISA titers for *Aotus* sera was determined as the X-axis intersection of the ELISA titration curve. Figure 8A: BVp42/MF59 (Group I); Figure 8B: BVp42/MTP-PE+MF59 (Group II); Figure 8C: BVp42/QS21 (Group III); Figure 8D: BVp42/ISA51 (Group IV).

Figure 9 shows the results of a T-cell proliferation assay of mononuclear cell cultures of *Aotus* vaccinated with BVp42 and either MF59 (I) or MTP-PE + MF-59 (II).

Figure 10A shows the results of intracellular cytokine producing cells of unstimulated cultures of *Aotus* vaccinated with BVp42 in adjuvants QS21 (III), MTP-PE+MF59 (II), or ISA51 (IV). IFNγ: interferon-gamma; IL-4: interleukin-4; IL-10: interleukin-10.

Figure 10B shows the results of intracellular cytokine producing cells of BVp42 stimulated cultures of *Aotus* vaccinated with BVp42 in adjuvants QS21 (III), MTP-PE+MF59 (II), or ISA51 (IV). IFNγ: interferon-gamma; IL-4: interleukin-4; IL-10:

interleukin-10.

Figures 11A-D show the parasitemia of *Aotus* monkeys immunized with BVp42 adjuvant preparations and administered with a lethal *P. falciparum* challenge. Figure 11A: BVp42/MF59; Figure 11B: BVp42/QS21; Figure 11C: BVp42/MTP-PE+MF59; Figure 11D: BVp42/ISA51.

Figure 12 shows the nucleotide sequence of the BVp42-M construct modified for optimized insect cell line expression (SEQ ID NO:6) and deduced amino acid sequence (SEQ ID NO:16).

Figure 13 shows the three-part construction of the p42-K coding region of the K1 allele of MSP-1 derived from the FVO strain of *P. falciparum*. Restriction sites used to ligate the fragments are underlined.

Figure 14 shows the DNA sequence of BVp42-K (SEQ ID NO:7) and deduced amino acid sequence (SEQ ID NO:8).

Figure 15 shows a silver stain of affinity chromatography purified BVp42-K. Lane 1: starting material; Lane 2: column filtrate; Lane 3: binding buffer flow through; Lane 4: washing buffer flow through; Lanes 5-9: purified protein aliquots 3-7. Samples were electrophoresed on an 11.5% polyacrylamide gel.

Figure 16 shows a western blot of affinity chromatography purified BVp42-K. Lane 1: starting material; Lane 2: column filtrate; Lane 3: binding buffer flow through; Lane 4: washing buffer flow through; Lanes 5-8: purified protein aliquots 3-6. Samples were electrophoresed on an 11.5% polyacrylamide gel.

Figure 17 shows a western blot of phenyl HIC and Ni-NTA chromatography purified BVp42-K. Lane 1: pooled staring material after phenyl HIC chromatography; Lane 2: column filtrate; Lane 3 is washing buffer flow through; Lanes 4-8: purified protein aliquots 3-7. Samples were electrophoresed on an 11.5% polyacrylamide gel.

Figures 18A and 18B show western blots of various Mabs with BVp42 (MAD20 allele) and BVp42-K (K1 allele). Numbers and letters above the lanes correspond to the specific monoclonal antibody used. Panel A: Lane 1: benchmark ladder; Lanes 2, 4, 6: BVp42-K; Lanes 3, 5, 7: BVp42. Panel B: Lane 1: benchmark ladder; Lanes 2, 4, 6, 8: BVp42-K; Lanes 3, 5, 7, 9: BVp42. Samples were electrophoresed on an 11.5% polyacrylamide gel.

Figure 19A and 19B show the results of N-glycosidase F digestions of BVp42-K, BVp42, and FVO MSP-1. All samples were electrophoresed on an 11.5% polyacrylamide gel. Panel A: silver stained gel: Lanes 1-2: BVp42-K; Lanes 3-4: BVp42; Lanes 5-6: FVO MSP-1. Lanes 1, 3, 5 were digested with 0.1 units of N-glycosidase F. Lanes 2, 4, 6 are controls. Samples were electrophoresed on an 11.5% polyacrylamide gel. Panel B: western blot: Lanes 1-2: BVp42-K; Lanes 3-4: BVp42; Lanes 5-6: FVO MSP-1. Lanes 1, 3, 5 were digested with 0.1 units of N-glycosidase F. Lanes 2, 4, 6 are controls.

Figure 20 shows a western blot of recombinant baculovirus infected insect cell supernatants incubated with tunicamycin. Lanes 1-3: BVp42-K; Lanes 4-6: BVp42. Lanes 1 and 4 are control wells without tunicamycin. Lanes 2 and 5 contain 2.5 μg/ml tunicamycin. Lanes 3 and 6 contain 5 μg/ml tunicamycin. Aliquots of cell supernatants were taken at 55 hours post-infection and electrophoresed on an 11.5% polyacrylamide gel.

DETAILED DESCRIPTION OF THE INVENTION

BVp42 antigen of *Plasmodium falciparum* gp195 induces antibodies that strongly, if not substantially completely, inhibit parasite growth and forms the basis of a vaccine. The antibodies are extensively crossreactive with different parasite strains, and have been found to strongly or completely inhibit parasite growth of heterologous parasites to the same degree as homologous parasites. BVp42, as described herein, is a variant of the natural p42 processing fragment of gp195 that has been recombinantly expressed in insect cells using a baculovirus expression vector. The term "BVp42" as used herein refers to the p42 amino acid sequence as characteristically produced in insect cells, in particular in Sf9 or High Five™/BTI-TN-5B1-4 cells. The amino acid sequence of p42 of different isolates is shown in Figure 6, *i.e.* amino acids nos. 1333 to 1726 in the FUP isolate, 1308 to 1701 in the MAD isolate, 1264 to 1640 in the Wellcome isolate, and 1255 to 1631 in the K1 isolate (the numbers refer to the amino acids of the precursor molecule, gp195). The term "p42" as used herein refers to the corresponding sequences in other isolates as well. A preferred embodiment of the invention includes only the amino acids Ala_{1333} to Ser_{1705} of the FUP isolate (Chang *et al.* Exp. Parasitol. 67:1), or the corresponding amino acids of other isolates (e.g. Ala_{1308} to Ser_{1680} of the MAD isolate (Mackay *et al.* 1985. EMBO J. 4:3823-3829), Ala_{1264} to Ser_{1619} of the Wellcome isolate (Holder *et al.* 1985. Nature 317:270-273), Ala_{1255} to Ser_{1610} of the K1 isolate (Mackay *et al.* 1984. EMBO J. 4:3823-3829)). (The numbering of these amino acids also corresponds to that shown in Figure 6.) The antigen of this embodiment preferably deletes the anchor sequence at the C- terminus of p42, allowing easier recovery of the product because it is secreted from the host cells.

The amino acid sequences of isolates bearing the same designation may vary somewhat, as may the DNA sequences coding for those isolates. Similarly, the numberings of the amino acid and DNA sequences in other publications may differ from the numberings shown herein. These and other aspects of the invention are more fully described below. For example, a particular amino acid and DNA sequence of a FUP isolate corresponding to amino acids Ala_{1333} to Ser_{1705} (SEQ ID NO:2) (as shown in Figure 6) is described in the examples below and has the sequences shown in Figure 7 (SEQ ID NO:1).

Accordingly, by "p42 polypeptide" herein is meant a polypeptide comprising a p42 amino acid sequence, including fragments and variants thereof, of the *Plasmodium* major merozoite surface protein (gp195). By "polypeptide" and grammatical equivalents herein are meant proteins, oligopeptides, and peptides, derivatives and analogs, including proteins containing non-naturally occurring amino acids and amino acid analogs, and peptidomimetic structures. The side chains may be in either the (R) or the (S) configuration. In a preferred embodiment, the amino acids are in the (S) or L-configuration. In some embodiments, for example when the p42 polypeptides are made synthetically, it may be desirable to utilize protein analogs to retard degradation by sample contaminants.

In a preferred embodiment, the p42 polypeptide is isolated. By "isolated polypeptide" herein is meant a polypeptide which has been identified and separated and/or recovered from a component of its natural environment. Contaminant components of its natural environment are materials which would interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to greater than 95% by weight of polypeptide as determined by the Lowry method, and most preferably more than 99% by weight, (2) to a degree sufficient to obtain at least about 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing conditions using Coomassie blue or, preferably, silver stain. Isolated p42 polypeptide includes the p42 polypeptide expressed by recombinant cells since at least one component of the polypeptide's natural environment will not be present. Ordinarily, however, isolated p42 polypeptide will be prepared by at least one purification step.

In a preferred embodiment, a p42 polypeptide comprises a native sequence p42 polypeptide. A "native sequence" p42 polypeptide comprises a polypeptide having the same amino acid sequence as a p42 polypeptide derived from nature. The term "native sequence" specifically encompasses naturally-occurring truncated or secreted forms (*e.g.*, an extracellular domain sequence), naturally-occurring variant forms (*e.g.*, alternatively

spliced forms) and naturally-occurring allelic variants and naturally-occurring *Plasmodium* species variants. In one embodiment of the invention, the native sequence p42 is a mature or full-length native sequence p42 polypeptide.

The p42 "extracellular domain" refers to a form of the p42 polypeptide which is essentially free of the transmembrane (Haldar et al. 1985. J. Biol. Chem. 260(8):4969-4974) and cytoplasmic domains. Ordinarily, a p42 polypeptide extracellular domain will have less than about 1% of such transmembrane and/or cytoplasmic domains and preferably, will have less than about 0.5% of such domains. It will be understood that any transmembrane domain(s) identified for the p42 polypeptides of the present invention are identified pursuant to criteria routinely employed in the art to identify that type of hydrophobic domain. In a preferred embodiment the transmembrane domain is identified as that portion of the p42 polypeptide that anchors the p42 polypeptide to a membrane. In an alternative embodiment, in the absence of the transmembrane domain, the p42 polypeptide is not anchored to a membrane and is therefore not associated with the cell in which the p42 polypeptide is expressed. Accordingly, in a preferred embodiment the p42 polypeptide extracellular domain comprises amino acids from about Ala_{1333} to about Ser_{1705} of the FUP isolate (SEQ ID NO:2) (Chang et al. Exp. Parasitol. 67:1), or the corresponding amino acids of other isolates (e.g. Ala_{1308} to Ser_{1680} of the MAD isolate (SEQ ID NO:4) (Mackay et al. 1985. EMBO J. 4:3823-3829), Ala_{1264} to Ser_{1619} of the Wellcome isolate (SEQ ID NO:5) (Holder et al. 1985. Nature 317:270-273), Ala_{1255} to Ser_{1610} of the K1 isolate (SEQ ID NO:3) (Mackay et al. 1984. EMBO J. 4:3823-3829).

In one embodiment, p42 polypeptides are identified by having substantial amino acid sequence homology with the amino acid sequences provided herein. In another embodiment, p42 polypeptide is identified as being encoded by a nucleic acid having substantial nucleic acid sequence homology with the nucleic acid sequences that are provided herein or with the nucleic acid sequences that encode the amino acid sequences provided herein. By "homology" herein is meant sequence similarity and identity with identity being preferred. Such sequence identity or similarity can be based upon the overall amino acid or nucleic acid sequence.

In a preferred embodiment, a polypeptide is a p42 polypeptide as defined herein if the overall sequence identity of the amino acid sequences of Figure 6 is preferably greater than about 60%, more preferably greater than about 70%, even more preferably greater than about 80% and most preferably greater than 90%. In some embodiments the sequence identity will be as high as about 93 to 95 or 98%.

As is known in the art, a number of different programs can be used to identify whether a

protein (or nucleic acid as discussed below) has sequence identity or similarity to a known sequence. Sequence identity and/or similarity is determined using standard techniques known in the art, including, but not limited to, the local sequence identity algorithm of Smith & Waterman. 1981. Adv. Appl. Math. 2:482, which is expressly incorporated by reference, by the sequence identity alignment algorithm of Needleman & Wunsch. 1970. J. Mol. Biol. 48:443, which is expressly incorporated by reference, by the search for similarity method of Pearson & Lipman. 1988. PNAS USA 85:2444, which is expressly incorporated by reference, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, WI), the Best Fit sequence program described by Devereux *et al.* 1984. Nucl. Acid Res. 12:387-395, all of which are expressly incorporated by reference, preferably using the default settings, or by inspection. Preferably, percent identity is calculated by FastDB based upon the following parameters: mismatch penalty of 1; gap penalty of 1; gap size penalty of 0.33; and joining penalty of 30, "Current Methods in Sequence Comparison and Analysis," Macromolecule Sequencing and Synthesis, Selected Methods and Applications, pp 127-149 (1988), Alan R. Liss, Inc., which is expressly incorporated by reference.

An example of a useful algorithm is PILEUP. PILEUP creates a multiple sequence alignment from a group of related sequences using progressive, pairwise alignments. It can also plot a tree showing the clustering relationships used to create the alignment. PILEUP uses a simplification of the progressive alignment method of Feng & Doolittle. 1987. J. Mol. Evol. 35:351-360; the method is similar to that described by Higgins & Sharp. 1989. CABIOS 5:151-153, all of which are expressly incorporated by reference. Useful PILEUP parameters include a default gap weight of 3.00, a default gap length weight of 0.10, and weighted end gaps.

Another example of a useful algorithm is the BLAST algorithm, described in Altschul et al., J. Mol. Biol. *215*, 403-410, (1990) and Karlin et al., PNAS USA 90:5873-5787 (1993), all of which are expressly incorporated by reference. A particularly useful BLAST program is the WU-BLAST-2 program which was obtained from Altschul et al., Methods in Enzymology, 266: 460-480 (1996); http://blast.wustl/edu/blast/ README.html], all of which are expressly incorporated by reference. WU-BLAST-2 uses several search parameters, most of which are set to the default values. The adjustable parameters are set with the following values: overlap span =1, overlap fraction = 0.125, word threshold (T) = 11. The HSP S and HSP S2 parameters are dynamic values and are established by the program itself depending upon the composition of the particular sequence and composition of the particular database against which the sequence of interest is being searched; however, the values may be adjusted to increase sensitivity.

An additional useful algorithm is gapped BLAST as reported by Altschul *et al.* Nucleic Acids Res. 25:3389-3402, which is expressly incorporated by reference. Gapped BLAST uses BLOSUM-62 substitution scores; threshold *T* parameter set to 9; the two-hit method to trigger ungapped extensions; charges gap lengths of *k* a cost of 10+*k*; X_u set to 16, and X_g set to 40 for database search stage and to 67 for the output stage of the algorithms. Gapped alignments are triggered by a score corresponding to ~22 bits.

A % amino acid sequence identity value is determined by the number of matching identical residues divided by the total number of residues of the "longer" sequence in the aligned region. The "longer" sequence is the one having the most actual residues in the aligned region (gaps introduced by WU-Blast-2 to maximize the alignment score are ignored).

In a similar manner, "percent (%) nucleic acid sequence identity" with respect to the coding sequence of the polypeptides identified herein is defined as the percentage of nucleotide residues in a candidate sequence that are identical with the nucleotide residues in the coding sequence of p42 polypeptide. A preferred method utilizes the BLASTN module of WU-BLAST-2 set to the default parameters, with overlap span and overlap fraction set to 1 and 0.125, respectively.

The alignment may include the introduction of gaps in the sequences to be aligned. In addition, for sequences which contain either more or fewer amino acids or nucleic acid residues than the sequences in the figures, it is understood that in one embodiment, the percentage of sequence identity will be determined based on the number of identical amino acid or nucleic acid residues in relation to the total number of residues. Thus, for example, sequence identity of sequences shorter than that shown in Figure 1, as discussed below, will be determined using the number of amino acids in the shorter sequence, in one embodiment. In percent identity calculations relative weight is not assigned to various manifestations of sequence variation, such as, insertions, deletions, substitutions, *etc.*

In one embodiment, only identities are scored positively (+1) and all forms of sequence variation including gaps are assigned a value of "0", which obviates the need for a weighted scale or parameters as described herein for sequence similarity calculations. Percent sequence identity can be calculated, for example, by dividing the number of matching identical residues by the total number of residues of the "longer" sequence in the aligned region and multiplying by 100. The "longer" sequence is the one having the most actual residues in the aligned region.

As will be appreciated by those skilled in the art, the sequences of the present invention may contain sequencing errors. That is, there may be incorrect nucleosides, frameshifts, unknown nucleosides, or other types of sequencing errors in any of the sequences; however, the correct sequences will fall within the homology and stringency definitions herein.

The p42 polypeptides of the present invention may be shorter or longer than the amino acid sequences shown in the figures. Thus, in a preferred embodiment, included within the definition of p42 polypeptides are portions or fragments of the amino acid sequences provided herein. In one embodiment, fragments of p42 polypeptides are considered p42 polypeptides if a) they share at least one antigenic epitope; b) have at least the indicated sequence identity; and/or c) preferably have p42 polypeptide immunologic activity as defined herein. The nucleic acids encoding the p42 polypeptides also can be shorter or longer than the sequences in the figures.

In addition, as is more fully outlined below, p42 polypeptides can be made that are longer than those depicted in the figures. For example, by the addition of an epitope or purification tags, the addition of other fusion sequences, or the elucidation of additional coding and non-coding sequences. As described below, the fusion of a p42 polypeptides to a polypeptide, such as a *flg5* leader polypeptide, is particularly preferred.

p42 polypeptides may also be identified as encoded by p42 nucleic acids which hybridize to the sequences depicted in the figures or to nucleic acid sequences that encode the amino acid sequences depicted in the figures, or the complement thereof, as outlined herein. Hybridization conditions are further described below.

In a preferred embodiment, p42 polypeptide must share at least one epitope or determinant with the full length protein. By "epitope" or "determinant" herein is meant a portion of a protein which will generate and/or bind an antibody. Thus, in most instances, antibodies made to smaller p42 polypeptides will be able to bind to the full length protein. In another embodiment, antibodies made to native p42 polypeptide will bind to smaller p42 polypeptides, provided that the smaller polypeptides contain an epitope found on the full-length polypeptide that is recognized by the antibodies made to native p42 polypeptide. Accordingly, in a preferred embodiment p42 polypeptide is immunogenic. By "immunogenic" or "immunogen" and grammatical equivalents herein is meant a substances that induces or evokes an immune response, such as a cell-mediated and/or humoral (antibody) immune response, in a mammal. In a preferred embodiment the immune response substantially reduces the symptoms and manifestations associated with plasmodium infection, as described herein.**~~BOLD~~

In the case of the nucleic acid, the overall sequence identity of the nucleic acid sequence is commensurate with amino acid sequence identity but takes into account the degeneracy in the genetic code and codon bias of different organisms. Accordingly, the nucleic acid sequence identity may be either lower or higher than that of the protein sequence. Thus the sequence identity of the nucleic acid sequence as compared to the nucleic acid sequence of the figures is preferably greater than 75%, more preferably greater than about 80%, particularly greater than about 85% and most preferably greater than 90%. In some embodiments the sequence identity will be as high as about 93 to 95 or 98%.

In a preferred embodiment, a p42 nucleic acid encodes a p42 polypeptide. As will be appreciated by those in the art, due to the degeneracy of the genetic code, an extremely large number of nucleic acids may be made, all of which encode the p42 polypeptides of the present invention. Thus, having identified a particular amino acid sequence, those skilled in the art could make any number of different nucleic acids, by simply modifying the sequence of one or more codons in a way which does not change the amino acid sequence of the p42 polypeptide.

In one embodiment, a p42 polypeptide is identified as being encoded by a nucleic acid that hybridizes under high stringency to the nucleic acids sequence shown in the figures, or their complement. High stringency conditions are known in the art; see for example Maniatis et al., Molecular Cloning: A Laboratory Manual, 2d Edition, 1989, and Short Protocols in Molecular Biology, ed. Ausubel, et al., both of which are hereby incorporated by reference. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences specifically hybridize at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, Techniques in Biochemistry and Molecular Biology-Hybridization with Nucleic Acid Probes, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993), which is expressly incorporated by reference. Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength, pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic acid concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m, 50% of the probes are occupied at equilibrium). Stringent conditions will be those in which the salt concentration is less than about 1.0 M sodium ion, typically about 0.01 to 1.0 M sodium ion concentration (or other salts) at about pH 7.0 to 8.3 and the temperature is at least about 30°C for short probes (e.g. about 10 to 50 nucleotides) and at least about 60°C for long probes (e.g. greater than about 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

In another embodiment, less stringent hybridization conditions are used; for example, moderate or low stringency conditions may be used, as are known in the art; see Maniatis and Ausubel, supra, and Tijssen, supra.

As used herein and further defined below, "nucleic acid" may refer to either DNA or RNA, or molecules which contain both deoxy- and ribonucleotides. The nucleic acids include genomic DNA, cDNA, and oligonucleotides including sense and anti-sense nucleic acids. Such nucleic acids may also contain modifications in the ribose-phosphate backbone to increase stability and half life of such molecules in physiological environments.

The nucleic acid may be double stranded, single stranded, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences depicted in the figures also include the complement of the sequence.

By the term "recombinant nucleic acid" herein is meant nucleic acid, originally formed *in vitro* or in a cell in culture, in general, by the manipulation of nucleic acid by endonucleases and/or exonucleases and/or polymerases and/or ligases and/or recombinases, to produce a nucleic acid not normally found in nature. Thus an isolated nucleic acid, in a linear form, or an expression vector formed *in vitro* by ligating DNA molecules that are not normally joined, are both considered recombinant for the purposes of this invention. It is understood that once a recombinant nucleic acid is made and reintroduced into a host cell or organism, it will replicate non-recombinantly, i.e., using the in vivo cellular machinery of the host cell rather than in vitro manipulations; however, such nucleic acids, once produced recombinantly, although subsequently replicated non-recombinantly, are still considered recombinant for the purposes of the invention.

Similarly, a "recombinant protein" is a protein made using recombinant techniques, i.e. through the expression of a recombinant nucleic acid as depicted above. A recombinant protein is distinguished from naturally occurring protein by at least one or more characteristics. For example, the protein may be isolated or purified away from some or all of the proteins and compounds with which it is normally associated in its wild type host, and thus may be substantially pure. For example, an isolated protein is unaccompanied by at least some of the material with which it is normally associated in its natural state, preferably constituting at least about 0.5%, more preferably at least about 5% by weight of the total protein in a given sample. A substantially pure protein comprises at least about 75% by weight of the total protein, with at least about 80% being

preferred, and at least about 90% being particularly preferred. The definition includes the production of a protein from one organism in a different organism or host cell. Alternatively, the protein may be made at a significantly higher concentration than is normally seen, through the use of an inducible promoter or high expression promoter, such that the protein is made at increased concentration levels. Alternatively, the protein may be in a form not normally found in nature, as in the addition of an epitope tag or amino acid substitutions, insertions and/or deletions, as discussed below.

Included in the definition of p42 polypeptides are p42 polypeptide variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding a p42 polypeptide, using cassette or PCR mutagenesis, scanning mutagenesis, gene shuffling or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant p42 polypeptide fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the p42 polypeptide amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed p42 polypeptide variants are screened for the optimal combination of desired activity. Techniques for making mutations at predetermined sites in DNA having a known sequence are well known. For example, the variations can be made using oligonucleotide-mediated site-directed mutagenesis [Carter et al., Nucl. Acids Res., 13:4331 (1986); Zoller et al., Nucl. Acids Res., 10:6487 (1987)], cassette mutagenesis [Wells et al., Gene, 34:315 (1985)], restriction selection mutagenesis [Wells et al., Philos. Trans. R. Soc. London SerA, 317:415 (1986)], all of which are expressly incorporated by reference, PCR mutagenesis, or other known techniques can be performed on the cloned DNA to produce the p42 polypeptide variant DNA. Scanning amino acid analysis can also be employed to identify one or more amino acids along a contiguous sequence. Among the preferred scanning amino acids are relatively small, neutral amino acids. Such amino acids include alanine, glycine, serine, and cysteine. Alanine is typically a preferred scanning amino acid among

this group because it eliminates the side-chain beyond the beta-carbon and is less likely to alter the main-chain conformation of the variant [Cunningham and Wells, Science, 244: 1081-1085 (1989), which is expressly incorporated by reference]. Alanine is also typically preferred because it is the most common amino acid. Further, it is frequently found in both buried and exposed positions [Creighton, The Proteins, (W.H. Freeman & Co., N.Y.); Chothia, J. Mol. Biol., 150:1 (1976), which are expressly incorporated by reference]. If alanine substitution does not yield adequate amounts of variant, an isoteric amino acid can be used. Screening of the mutants or variants is done using assays of p42 polypeptide activities and/or properties as described herein.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the p42 polypeptide are desired, substitutions are generally made in accordance with the following chart:

Chart I

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect the structure of the

polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl, is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

The variants typically exhibit the same qualitative biological activity and will elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the p42 polypeptide as needed, for example, to increase the immunogenicity of the p42 polypeptide. Alternatively, the variant may be designed such that the biological activity of the p42 polypeptide is not altered. For example, glycosylation sites may be added, altered or removed. p42 polypeptide may be designed to add phosphorylation sites.

p42 polypeptide fragments may be prepared by any of a number of conventional techniques. Desired peptide fragments may be chemically synthesized. An alternative approach involves generating p42 polypeptide fragments by enzymatic digestion, e.g., by treating the protein with an enzyme known to cleave proteins at sites defined by particular amino acid residues, or by digesting the DNA with suitable restriction enzymes, expressing the digested DNA and isolating the desired fragment. Yet another suitable technique involves isolating and amplifying a DNA fragment encoding a desired polypeptide fragment, by polymerase chain reaction (PCR). Oligonucleotides that define the desired termini of the DNA fragment are employed at the 5' and 3' primers in the PCR. Preferably, p42 polypeptide fragments share at least one biological and/or immunological activity with the native polypeptides shown in the figures.

In another aspect of the invention, as noted above, we have found that when a p42 amino acid sequence is expressed in insect cells, a pure product can be obtained without degradation or cleavage if DNA coding for a MAD allele sequence is employed. An amino acid sequence of the p42 antigen is considered to be of the MAD allele if it corresponds to the parts of gp195 of the MAD *Plasmodium falciparum* isolate which are dimorphic as compared with the amino acid sequences of the Wellcome and K1 isolates (Tanabe *et al.* 1987. J. Mol. Biol. 195:273). For example, gp195 of the FUP isolate is of the MAD allele (Chang *et al.* 1989. Proc. Natl. Acad. Sci. USA 86:6343; Chang *et al.*

1988. Exp. Parasitol. 67:1) (FUP isolate p42 amino acid sequence is as well).

The method for producing BVp42 according to this aspect of the invention involves infecting an insect cell host with a recombinant baculovirus vector, that vector containing DNA coding for a p42 amino acid sequence of surface protein gp195 of the *Plasmodium falciparum* MAD allele, operably linked to a baculovirus polyhedron promoter. Production of BVp42 with the sequence of the MAD allele, preferably of the FUP isolate sequence, results in pure, undegraded antigen.

A high level of inhibition of parasite growth is exhibited by anti-BVp42 sera. As noted above, strong inhibition of parasite growth by sera of monkeys vaccinated with Mab 5.2 affinity purified parasite gp195 correlates with the ability to induce substantially complete protection against infection. Other types of purification systems also can be used as known in the art, such as, molecular exclusion chromatography, phenyl HIC. Affinity chromatography also may employ a specific sequence or moiety either native or added to p42 wherein the sequence or moiety specifically binds to a compound or chemical such as in Ni-NTA chromatography, or an epitope can be added or fused to the p42 protein which is purifed by affinity chromatography with an antibody the binds to the added epitoped.

Without wishing to be bound by any theory of the invention, our results provide new insights into the interpretation of the vaccination study discussed above utilizing parasite gp195 that was affinity purified using Mab 5.2. As shown herein, utilization of this Mab (now found by us to be specific for a conformational epitope located within the p42 processing fragment) for affinity purification of parasite gp195 results in an antigen preparation that contains the gp195 precursor but that is also highly enriched for several C-terminal processing fragments, including p42. Based on the analysis of the specificity of anti-parasite gp195 antibodies utilizing the BVp42 polypeptide, a majority of antibodies produced by immunization with Mab 5.2 affinity purified parasite gp195 is specific for the C-terminal p42 processing fragment. The importance of p42 epitopes in immunity is consistent with the strong inhibition of parasite growth obtained with anti-BVp42 sera. There have been reports of several Mabs specific for this region which inhibit *in vitro* parasite growth (Pirson *et al.* 1985. J. Immunol. 134:1946; Blackman *et al.* 1990. J. Exp. Med. 172:379). Thus, it is possible that the exceptional level of protection achieved in the previous vaccination study was due to the focusing of the immune response on C-terminal epitopes which appear to serve as targets of functional effects such as the direct inhibition of parasite growth.

A study of the influence of MHC genes on immunological responsiveness to gp195 had found that a variety of congenic mouse strains were capable of producing antibodies

against gp195 (Chang *et al.* 1989. Proc. Natl. Acad. Sci. USA 86:6343). We have detected BVp42-specific antibodies in seven congenic mouse strains immunized with purified, parasite gp195, indicating that individuals of many H-2 haplotypes are capable of recognizing epitopes within the smaller, p42 region of gp195. Thus, BVp42 can likely be used as a vaccine antigen in hosts of diverse genetic makeup.

Techniques known to one skilled in the art for expressing foreign genes in insect host cells can be used to practice the invention. Methodology for expressing polypeptides in insect cells is described, for example, in Summers and Smith. 1986. A Manual of Methods for Baculovirus Vectors and Insect Culture Procedures, Texas Agricultural Experimental Station Bull. No. 7555, College Station, TX; Luckow. 1991. In Prokop *et al.*, Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors' Recombinant DNA Technology and Applications, 97-152; and in U.S. Patent No. 4,745,051, all hereby incorporated by reference in their entirety. The techniques are summarized below.

To make BVp42, or an effective immunogenic part thereof, a polyhedron shuttle vector is used to shuttle all or part of the p42 coding sequence into a nuclear polyhedrosis virus. This vector contains the promotor of the polyhedron gene of a nuclear polyhedrosis virus and an available cloning site for the insertion of a selected gene such that the selected gene is under transcriptional control of the polyhedron promotor. Thus, the shuttle vector is engineered to contain the p42 sequence operably linked to a baculovirus polyhedrin promoter.

For example, the p42 sequence can be inserted into a commonly used polyhedron transfer vector, such as pAC373, to produce a recombinant shuttle vector for introducing foreign genes into a nuclear polyhedrosis viruses, such as *Polyhedron californica* nuclear polyhedrosis virus (AcMNPV), resulting in a recombinant viral expression vector capable of expressing the gene encoding for p42 in a host insect cell. pAC373 contains a deletion of the sequence between -8 (8 bases upstream from the polyhedron ATG and approximately 40 bases downstream from the polyhedron transcriptional start site) and the natural BamHI sites at nucleotide +171 (Luckow *et al.* 1988. Trends in the Development of Baculovirus Expression Vectors, Biotechnology, 6:47) resulting in a construct which can be used to express full-length gene which contains an internal ATG (N-formyl methionine initiation).

Any p42 coding DNA can be used to make the expression vector. While natural DNA sequences of various *Plasmodium falciparum* isolates are described herein, it is within ordinary skill in the art to vary those sequences. Thus, non-natural DNA sequences,

different from the particular ones listed herein, can be used effectively in practicing the invention. For example, given the degeneracy of the genetic code DNA sequences encoding a protein can be modified to optimize expression for particular organisms and/or cells types, such as, tailoring the codon usage to those codons that are preferred by a given expression system, without modifying the desired amino acid sequence of the expressed protein.

Similarly, non-natural amino acid sequences can be coded for by the DNA used in the transfer vector in order to obtain functional products that obtain the advantages of the invention.

The C-terminus of BVp42 is preferably truncated, as discussed above, to remove the hydrophobic tail sequence (*i.e.*, membrane anchor) to allow for protein secretion. For example, the entire anchor sequence e.g. amino acids 1708-1725 of the FUP isolate and corresponding amino acids of other isolates, can be deleted. An embodiment discussed in the examples below deletes an additional two amino acids at the N-terminus of the anchor sequence.

Thus it is within the scope of the invention to delete portions of the p42 amino acid sequence which do not affect the beneficial result obtained with the BVp42 products exemplified herein.

Transfer of the hybrid DNA to an expression vector is accomplished by transfection of the host insect cell, e.g. *Spodoptera frugiperda* (sf) cells, with a mixture of both the recombinant transfer plasmid DNA and wild type nuclear polyhedrosis virus (MNPV). The transfected plasmid DNA recombines with the homologous sequences in the wild-type baculovirus genome to produce a viral genome that carries an integrated copy of the foreign gene.

The recombinant expression vector, comprising the hybrid polyhedron-p42 gene incorporated in the MNPV genome is then selected from the mixture of nonrecombinant and recombinant baculoviruses. For example, the supernatant containing the mixture of wild-type and recombinant budded viruses is collected, clarified by centrifugation and used for subsequent plaque assays. The preferred means of selection is by visual screening for the absence of viral occlusion bodies or by plaque hybridization with nucleic acid coding for p42, such as described in Kiefer *et al.* 1991. Growth Factors 5:115-127.

Expression of the p42 gene is accomplished by infecting susceptible host insect cells,

such as sf cells, with the recombinant baculovirus p42-expression vector in an appropriate medium for growth. Propagation of the baculovirus p42-expression vector is achieved in the insect cells through replication and assembly of infectious virus particles.

Various nuclear polyhedrosis viruses can be employed in making expression vectors for use in the invention. Suitable viruses include, but are not limited to, *Polyhedron californica* nuclear polyhedrosis virus (AcMNPV), *Spodoptera frugiperda* nuclear polyhedrosis virus, *Choristoneura fumiferana* nuclear polyhedrosis virus, *Spodoptera littoralis* nuclear polyhedrosis virus, or *Trichoplusia ni* nuclear polyhedrosis virus, all known in the art and commonly available.

Suitable insect host cells for use in the invention include, but are not limited to, Sf9 (*Spodoptera frugiperda*), *Spodoptera exiaua*, *Choristoneura fumiferana*, *Trichoplusia ni*, and *Spodoptera littoralis*, *Drosophila* and other cells known in the art.

The polypeptide may be produced as either a fusion product, such as a heterologous protein containing part baculovirus amino acid sequence fused to *Plasmodium falciparum* sequence, or the product may merely contain a *Plasmodium falciparum* sequence. Methods of making both types of proteins are well known to one skilled in the art.

Accordingly, in a preferred embodiment, the invention provides fusion polypeptides comprising a, first, p42 polypeptide and at least a second polypeptide, wherein the second polypeptide is a p42 polypeptide, as defined herein, or a heterologous polypeptide. The p42 polypeptide and second polypeptide are fused covalently or non-covalently, with covalently being preferred. As will be appreciated by those in the art, the fusion polypeptides of the invention can be configured in a variety of ways. In one embodiment, the p42 polypeptide is fused to the carboxy terminus of the second polypeptide. Alternatively, the p42 polypeptide is fused to the amino-terminus of the second polypeptide. In another alternative embodiment, the p42 polypeptide is at a position internal to the carboxy and amino termini or the second polypeptide. Accordingly, the p42 polypeptide and the second polypeptide are joined to produce linear fusions or branched fusions in any manner as the biology and activity permits, although in general, N- or C-terminal fusions are preferred to internal fusions.

In general, the fusion polypeptides of the invention can be made either recombinantly or synthetically.

In a preferred embodiment, the p42 and second polypeptides are attached through the use of functional groups on each that can then be used for attachment. Preferred functional

groups for attachment are amino groups, carboxy groups, oxo groups and thiol groups. These functional groups can then be attached, either directly or indirectly through the use of a linker. Linkers are well known in the art; for example, homo-or hetero-bifunctional linkers as are well known (see 1994 Pierce Chemical Company catalog, technical section on cross-linkers, pages 155-200, incorporated herein by reference). Preferred linkers include, but are not limited to, alkyl groups (including substituted alkyl groups and alkyl groups containing heteroatom moieties), with short alkyl groups, esters, amide, amine, epoxy groups and ethylene glycol and derivatives being preferred, with propyl, acetylene, and C2 alkene being especially preferred. Suitable crosslinking agents include, *e.g.*, 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl) dithio]propioimidate. Accordingly, at least one amino acid that reacts with a crosslinking agent may be added to a p42 polypeptide or the second polypeptide antibody by insertion and/or substitution to facilitate crosslinking.

In a preferred embodiment, the p42 and second polypeptides are crosslinked to a third molecule, which accordingly provides a scaffold for linking the p42 and second polypeptides. Suitable scaffolds include, for example, peptides, carbohydrates, nucleic acids, lipids, small organic molecules and the like. The crosslinkers function to join the p42 and second polypeptide and preferably allow each component to function without interference from the other component or the crosslinker.

In one embodiment, linear fusions are preferred. Accordingly, the p42 polypeptide is directly fused either to the amino terminus, carboxy terminus, and/or is internal to the termini of the second polypeptide. In a preferred embodiment, linkers, spacers, or adapters comprised of amino acids are used to join the p42 polypeptide and second polypeptide. In some embodiments, the fusion nucleic acid optionally encodes linkers, crosslinkers, spacers, or adapters, as needed. The number of amino acids comprising the linker can be determined by routine experimentation by a skilled artisan. The linkers comprising a sufficient number of amino acids such that the p42 and second polypeptides function without interference from the other. Accordingly, amino acids that comprise the linker preferably do not substantially alter biological activity of the p42 or second polypeptide.

For example, useful linkers include glycine polymers $(G)_n$, glycine-serine polymers (including, for example, $(GS)_n$, $(GSGGS)_n$ and $(GGGS)_n$, where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers such

as the tether for the shaker potassium channel, and a large variety of other flexible linkers, as will be appreciated by those in the art. Glycine and glycine-serine polymers are preferred since both of these amino acids are relatively unstructured, and therefore may be able to serve as a neutral tether between components. Glycine polymers are the most preferred as glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (see Scheraga, Rev. Computational Chem. III73-142 (1992), expressly incorporated by reference). Secondly, serine is hydrophilic and therefore able to solubilize what could be a globular glycine chain. Third, similar chains have been shown to be effective in joining subunits of recombinant proteins such as single chain antibodies.

In a preferred embodiment, either or both p42 and the second polypeptide of the invention can comprise additional components or may be modified in other ways. For example, modification of the fusion polypeptides include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the "amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983), expressly incorporated by reference], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the p42 and second polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence of the fusion polypeptide components, and/or adding one or more glycosylation sites that are not present in the native sequences.

Addition of glycosylation sites may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the fusion polypeptide sequence (for O-linked glycosylation sites). The alteration also may be made, for example, by the addition of, or substitution by one or more Asn-Xaa-Ser/Thr sites (Xaa = any amino acid; for N-linked glycosylation sites) in the fusion polypeptide sequence. The fusion polypeptide amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the p42 polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the fusion

polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, CRC Crit. Rev. Biochem., pp. 259-306 (1981), all of which are expressly incorporated by reference.

Removal of carbohydrate moieties present on p42 or the fusion polypeptide may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981), all of which are expressly incorporated by reference. Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987), expressly incorporated by reference.

Another type of covalent modification ofp42 or the fusion polypeptide comprises linking the p42 or fusion polypeptide to one of a variety of nonproteinaceous polymers, *e.g.*, polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337, all of which are expressly incorporated by reference.

In one embodiment, the p42 polypeptide is fused to an epitope tag which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the p42 polypeptide but may be incorporated as an internal insertion or substitution as the biological activity permit. The presence of such epitope-tagged forms of a p42 polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the p42 polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988), which is expressly incorporated by reference]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985), which is expressly incorporated by reference]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990), which is expressly incorporated by reference]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210

(1988), which is expressly incorporated by reference]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992), which is expressly incorporated by reference]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990), which is expressly incorporated by reference] and the histidine tag and metal binding sites (Smith, Ann. NY. Acad. Sci., 646:315-321 (1991), which is expressly incorporated by reference], with the Flag and histidine tag being preferred.

In a preferred embodiment, nucleic acid encoding a leader sequence can be included in the expression vector and fused to the amino terminus of the p42 polypeptide to facilitate sorting through the endoplasmic reticulum and proper folding of the polypeptide product. For example, *flg*5 cDNA encoding a leader sequence can be inserted 5' to the p42 coding region using conventional techniques. The *flg*5 leader is described in Kiefer *et al.* 1991. Growth Factors 5:115-127. It is also disclosed in the Genebank and EMBL data bases (accession no. M60485). In addition, the *flg*5 DNA sequence used in a construct described in the examples below is shown in Figure 7. The final purified product according to the invention may include a leader sequence, or the sequence may be cleaved during expression. Amino-terminal amino acid sequencing indicates that the polypeptide is appropriately cleaved.

The many variations in techniques for expressing and isolating BVp42 will be apparent to one skilled in the art. For example, promoters known in the art can be modified to alter expression. In addition, the number and composition of the nucleotides in the region between the promoter and start of the open reading frame can be modified to alter expression.

The BVp42 product can be conventionally purified, such as, in part, by affinity chromatography with a Mab specific for natural p42. Mab 5.2 is one such antibody. In another embodiment, BVp42 can be purified, for example, by the addition of a tag to the p42 polypeptide as described herein, preferably at the carboxy terminus, and purified by the appropriate affinity purification methods.

The BVp42 obtained is a variant of naturally occurring p42 that results from characteristic post-translational processing occurring in insect cells, especially Sf9 cells. Potential sites for post-translational modifications are shown in Figure 6.

BVp42 has been found to be highly immunogenic in rabbits. High antibody titers against the immunogen can be obtained which meet or exceed titers of animals immunized with purified parasite gp195. ELISA titers were found similar in assays utilizing plates coated

with either purified, parasite gp195 or BVp42. More importantly, high titers were obtained when anti-BVp42 antibodies were reacted with purified, parasite gp195 in an ELISA and in an indirect immunofluorescence assay with schizonts and merozoites. IFA titers obtained after the fourth immunization with BVp42 reached levels exceeding those obtained by immunization with purified, parasite gp195.

Yeast produced p42 (Yp42) consisting of the same p42 sequence (amino acids nos. 1333 to 1705 in the FUP isolate) was found to be less immunogenic than BVp42, inducing lower antibody titers against the immunogen. In addition, the cross-reactivity of anti-Yp42 antibodies with parasite gp195 in the ELISA was much lower than cross-reactivity of anti-BVp42 antibodies. Yp42 also induced much lower IFA titers than BVp42, and statistically insignificant levels of parasite inhibition.

Immunoblotting studies demonstrated that most of the anti-BVp42 and anti-parasite gp195 antibodies produced are specific for disulfide-dependent, conformational epitopes present on the same set of gp195 processing fragments.

Accordingly, once made and purified, if necessary, the p42 polypeptides and p42 polypeptide fusions are useful in a number of applications, for example, in the induction of an anti-plasmodium immune response or treatment of a disease state in a patient or individual. The p42 polypeptides and fusions thereof of the present invention can be use alone or in combination with other therapeutic agents or carriers. In a preferred embodiment, the p42 polypeptides are employed as a vaccine.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) the targeted pathologic condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

"Chronic" administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. "Intermittent" administration is treatment that is not consecutively done without interruption, but rather is cyclic in nature.

By "patient" or "individual" herein refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, cats, cattle, horses, sheep, pigs, goats, rabbits, etc. Preferably, the mammal is human.

Administration "in combination with" one or more further therapeutic agents or carriers includes simultaneous (concurrent) and consecutive administration in any order.

In a preferred embodiment, an adjuvant should be included with the BVp42 in the vaccine of the invention in order to enhance the immune response. Such adjuvants include Freund's complete adjuvant, B30-MDP, LA-15-PH, (Hui *et al.* 1990. Vaccine. Cold Spring Harbor Press. 477-483; Hui *et al.* 1991. Infection and Immunity 59:1585-1591; Hui *et al.* 1991 J. Immunol. 147:3935-3941), Freund's incomplete adjuvant, saponin, aluminum hydroxide, MF59, MTP-PE, QA-21, ISA51 or other available adjuvants or adjuvant combinations. Freund's complete adjuvant is not generally used clinically for human vaccines.

In a preferred embodiment, a pharmaceutically acceptable carrier is included in the therapeutic formulation, preferably a vaccine. Such carriers are well known to one skilled in the art and can be formulated according to known methods to prepare pharmaceutically useful compositions. Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional physiologically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980), which is expressly incorporated by reference), in the form of lyophilized formulations or aqueous solutions. Acceptable carriers, excipients or stabilizers are nontoxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone, amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN™, PLURONICS™ or PEG.

The formulations to be used for *in vivo* administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes, prior to or following lyophilization and reconstitution.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

The route of administration is in accord with known methods, e.g. injection or infusion by

intravenous, intraperitoneal, intramuscular, intraarterial or intralesional routes, or by sustained release systems.

Dosages and desired drug concentrations of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary physician. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti, J. and Chappell, W. "The use of interspecies scaling in toxicokinetics" In Toxicokinetics and New Drug Development, Yacobi et al., Eds., Pergamon Press, New York 1989, pp. 42-96, which is expressly incorporated by reference.

When *in vivo* administration of a fusion polypeptide is employed, normal dosage amounts may vary from about 10 ng/kg to up to 100 mg/kg of mammal body weight or more per day, preferably about 1 μg/kg/day to 10 mg/kg/day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Pat. Nos. 4,657,760; 5,206,344; or 5,225,212, which are expressly incorporated by reference. It is anticipated that different formulations will be effective for different treatment compounds and different disorders, that administration targeting one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue.

Where sustained-release administration of a polypeptide is desired in a formulation with release characteristics suitable for the treatment of any disease or disorder requiring administration of the polypeptide, microencapsulation of the polypeptide is contemplated. Microencapsulation of recombinant proteins for sustained release has been successfully performed with human growth hormone (rhGH), interferon- (rhIFN-), interleukin-2, and MN rgp120. Johnson et al., Nat. Med., 2:795-799 (1996); Yasuda, Biomed. Ther., 27:1221-1223 (1993); Hora et al., Bio/Technology, 8:755-758 (1990); Cleland, "Design and Production of Single Immunization Vaccines Using Polylactide Polyglycolide Microsphere Systems," in Vaccine Design: The Subunit and Adjuvant Approach, Powell and Newman, eds, (Plenum Press: New York, 1995), pp. 439-462; WO 97/03692, WO 96/40072, WO 96/07399; and U.S. Pat. No. 5,654,010, all of which are expressly incorporated by reference.

The sustained-release formulations of polypeptides were developed using poly-lactic-coglycolic acid (PLGA) polymer due to its biocompatibility and wide range of biodegradable properties. The degradation products of PLGA, lactic and glycolic acids,

can be cleared quickly within the human body. Moreover, the degradability of this polymer can be adjusted from months to years depending on its molecular weight and composition. Lewis, "Controlled release of bioactive agents from lactide/glycolide polymer," in: M. Chasin and R. Langer (Eds.), Biodegradable Polymers as Drug Delivery Systems (Marcel Dekker: New York, 1990), pp. 1-41, which is expressly incorporated by reference.

The vaccine preferably contains from about 0.1 to 5 mg in about a 0.1 ml to about 1.0 ml dose of BVp42. The vaccine can be administered in one or more doses, the amount administered being adjusted to correspond with the number of inoculations, either together or over a period of time. Administration can be carried out conventionally, preferably parenterally. ~~**ASK TOMORROW~~

In a preferred embodiment, the vaccine preferably induces an immune response that substantially reduces the symptoms and manifestations associated with plasmodium infection, such as, the onset, the severity, and/or the duration of illness. The symptoms and manifestations include, for example, parasitemia, fever and chills which begin irregularly and later become synchronous in a non-immune individual. In a most preferred embodiment the immune response prevents overt or clinical disease.

In a preferred embodiment, the vaccine substantially delays the onset of a symptom in comparison to a non-vaccinated individual following infection at a dose that occurs normally in nature. Preferably, the symptom is parasitemia. Accordingly, the vaccine delays the onset of a symptom by about 2 days, preferably reduces onset of a symptom by about 4 days, more preferably by about 8 days, even more preferably by about 16 days or higher.

In a preferred embodiment, the vaccine substantially reduces parasitemia in comparison to a non-vaccinated individual following infection at a dose that occurs normally in nature. Accordingly, the vaccine reduces parasitemia by about 2 fold, preferably reduces parasitemia by about 10 fold, more preferably by about 100 fold, even more preferably by about 1,000 fold. In an even more preferred embodiment, parasitemia is reduced by 10,000 fold or higher and parasites can not be detected.

The present invention is further described in the examples below. The examples are for illustration purposes, and are not intended to limit the scope of the invention. All patents, patent applications, and publications and references cited therein are hereby expressly incorporated by reference in their entirety.

EXAMPLES

Example 1

Production and Purification of Baculovirus p42 (BVp42)

The Uganda Palo Alto (FUP) *Plasmodium falciparum* p42 coding region from Ala_{1333} to Ser_{1705}, was cloned into a *Polyhedron californica* nuclear polyhedrosis virus (AcMNPV) polyhedrin promoter regulated expression system (Luckow. 1988. Biotechnology 6:47).

Figure 1 shows, schematically, the sequences that were cloned into a BVp42 encoding shuttle vector. The coding sequence, shown at top, included the *flg*5 leader (Flg_L, solid box) fused to the FUP isolate p42 coding region from Ala_{1333} to Ser_{1705}. The BamHI fragment finally obtained was cloned into a AcMNPV transfer plasmid, shown at the bottom of the figure. The leader sequence for *flg*5 has been shown to direct secretion of the mature *flg*5 protein (Kiefer *et al.* 1991. Growth Factors 5:115-127). When fused to p42 coding DNA in the baculovirus expression system, the mature protein is also found to be secreted from the insect cells. "RV" is an EcoRV restriction site. The construct was made as follows.

Oligomers encoding the *flg*5 leader (Kiefer *et al. supra*), 5' portion of FUP isolate p42 from Ala_{1333} to the HindIII site at amino acid Arg_{1362} and BamHI sites were made on an automated DNA synthesizer (Applied Biosystems, Foster City, CA), kinased, annealed, and ligated. The overlapping oligomers consisted of six 43-mers, two 46-mers and two 19-mers. After digestion with BamHI, the ligation product was ligated into BamHI-digested and phosphatased pAB114, a HindIII/SalI deletion of pBR322 containing a BamHI linker (Barr *et al.* 1988. J. Biol. Chem. 263:16471). The HindIII/SalI fragment of p42 from a pAB125 ADH_2-GAPDH alpha-factor construct (Hui *et al.* 1991. J. Immunol. 147:3935-3941), which includes an in-frame stop codon following Ser_{1705}, was ligated in-frame into this pAB114 vector to yield a *flg*5 leader fused to full-length p42 flanked by unique BamHI sites. The BamHI fragment was subsequently cloned into the BamHI site of the AcMNPV transfer plasmid pAc373 (Luckow. 1988. Biotechnology 6:47). The recombinant shuttle vector obtained was then used to transfer the coding sequence into AcMNPV by homologous recombination through transfection of sf9 cells (Summers *et al.* 1986. Tex. Agr. Exp. Station Bull. No. 7555, College Station, TX). Recombinant viruses were initially identified visually by occlusion negative phenotype and plaque hybridization with the BamHI fragment labeled by the oligomer primed extension method (Feinberg *et al.* 1984. Anal. Biochem. 137:266). The recombinant AcMNPV expression vector obtained, Flg5LFUP42AcNPV, was deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA 20852, on January 28, 1992 and has been assigned accession number VR2354. Infection of sf9 cells was carried out conventionally per Summers *et al.* 1986. *supra*.

Protein expression was initially monitored in roller bottles. Production was then scaled up to 0.5 liter batches in 2.8 liter Fernbach shaker flasks. Culture supernatants were concentrated 10 to 50-fold by tangential flow ultrafiltration (Amicon). Recombinant BVp42 was purified from the concentrated supernatant by the affinity chromatography technique utilized to purify parasite gp195 as described by Siddiqui *et al.* 1987.

Figure 2A shows silver stains (lane 1) and immunoblots (lane 2) of the affinity chromatography purified BVp42. The silver stained antigens were electrophoresed in SDS polyacrylamide gels. The immunoblots were reacted with a rabbit anti-parasite gp195 serum pool.

A single band migrating at approximately 44 kDa was observed in the silver stain. In the immunoblots, the purified BVp42 displayed major immunoreactive species at the positions of the major protein bands, accompanied by minor reactivities with proteins of higher and lower molecular weight.

The DNA sequence of the BVp42 construct from BamHI to SalI is shown in Figure 7, the numbering starting with the first nucleotide of BamHI and ending with the last nucleotide of SalI. A restriction map is shown schematically at the top of the figure and the same sites are shown adjacent to the corresponding sequences below.

Edman sequence of the expressed protein of BVp42 resulted in a main N-terminal sequence consistent with the expected p42 N-terminus: ISVTMDNILS (SEQ ID NO:9) The yield of this sequence was lower than expected, suggesting that some of the material may have a blocked N-terminus. A minor sequence corresponding to the N-terminus of bovine serum albumin (BSA) was also detected, indicating that there was a trace amount of BSA in the preparation.

The nucleotide sequence of the original BVp42 construct was modified to optimize for insect cell line (High Five, *Trichoplusia ni*) codon preferences, distance between the promoter sequence and translation initiation codon, and preferred nucleotide composition of the sequences immediately before the initiation codon. The modified construct has been designated BVp42-M and the nucleotide sequence of this construct is shown in Figure 12.

Example 2

Production and Purification of Baculovirus p42-K

The K1 type (p42-K) of the p42 antigen was constructed using the Vietnam-Oak Knoll *P. falciparum* isolate (FVO). There are three parts to this construct: a leader sequence, the

p42-K coding region and the histidine tag (Figure 13). Restriction sites were incorporated into the primers to enable a "sticky-end" ligation of the three fragments. The leader sequence was altered from the original p42-M sequence such that three adenines were added three bases prior to the start site to optimize the codon preference for baculovirus and insect cells as well as the distance between the promoter sequence and the methionine start codon (Ranjan *et al.* 1995. Virus Genes 9(2):149-153). Primers containing NarI and PstI restriction site sequences were used to amplify the 1,065 base pair p42-K coding region corresponding to the Ala_{1349} to Ser_{1723} (as numbered by Miller *et al.* 1993. Mol. Biochem. Parasitol 59(1):1-14.) of MSP-1 from genomic *P. falciparum* DNA. Primers containing BamHI and NarI restriction site sequences were used to amplify the 91 base pair leader sequence. Oligonucleotides containing PstI and KpnI restriction site sequences were made to generate the 25 base pair histidine tag. All primers and oligonucleotide sequences used for the p42-K constructs are shown in Table 1.

Table 1

PCR Primer Sequences used in the Construction of p42-K

Primer Name	Sequence (5' to 3')
Leader F (SEQ ID NO:10)	ATTGGATCCACT**AAA**ATGTGGAGCTGGAAG
Leader R (SEQ ID NO:11)	TATGGCGCCCGCGGTGCAGAGTGTGGCTGT
p42 F (SEQ ID NO:12)	TTAGGCGCCGCAGTAACTCCTTCCGTAATT
p42 R (SEQ ID NO:13)	TAACTGCAGAAAATACCATCGAAAAGT
His F (SEQ ID NO:14)	TAACTGCAGTCATCATCATCATCATCATTAATAAGGTACCGAG
His R (SEQ ID NO:15)	ATAGGTACCTTATTAATGATGATGATGATGATGACTGCAGTTA

Underlined sequences represent restriction sites.

Bold letters represent changes to the leader sequence.

The leader sequence of the BVp42 constructs and the p42-K coding region from FVO malaria parasite DNA were amplified by a high fidelity PCR reaction containing both Taq and Pwo DNA polymerases. This allows for the cloning of the leader and p42-K coding region into the transfer vector without mutations, since Pwo DNA polymerases contain 3' to 5' exonuclease proofreading activity. PCR reaction mixtures were brought up to 100 microliters with 200 micromolar of each dNTPs (Gibco BRL), 0.4 micromolar primers, 10 microliters of DNA template, 0.75 units of high fidelity polymerase (Roche Molecular Biochemicals, Indianapolis, IN) and 1X high fidelity reaction buffer (Roche Molecular Biochemicals). In order to amplify the leader sequence 1 cycle of 95°C 5min., and 35 cycles of 95°C 1 min., 56°C 1 min., and 72°C 1 min. were used. For the amplification of the p42-K coding region, 1 cycle of 95°C 5 min, and 35 cycles of 95°C 1 min., 56°C 1

min., and 72°C 2 min. were used. The 1.1 kb p42-K PCR product was electrophoresed on a 1% Sea Plaque GTG (FMC) low melt agarose gel in 1X TAE and purified using the Geneclean III Kit (Bio101, Vista, CA). The 90 base pair leader PCR product was electrophoresed on a 2% BioGel (Bio101) low melt agarose gel designed for small DNA fragments, excised, and purified using the Mermaid Spin Kit (BIO 101, Inc., 1070 Jushua Way, Vista, CA 92083). To construct the histidine tag, the His F and His R oligonucleotides were diluted to 5 μM in dH_20. To anneal the oligonucleotides, equal volumes were heated to 37°C for 10 min. and at room temperature for 20 min.

The leader sequence, MSP-1 p42-K coding region and histidine tag were restriction enzyme digested, purified by Geneclean III or Mermaid Spin Kit as needed and ligated into pUC18 (Figure 13). The ligation was used to transform competent bacteria prepared as described by Sambrook et al. (1989). Plasmids from colonies were isolated and analyzed. Once a plasmid with the insert was identified as being in the correct orientation, the insert was subcloned into the transfer vector pAC373 and operably linked to the polyhedron promoter. Sequencing of the p42-K construct revealed a minor discrepancy when compared to the published MSP-1 sequence (Miller *et al.* 1993. Mol Biochem Parasitol. 59(1):1-14.) A nucleic acid base mutation occurred at position 670 of the p42-K coding region, which resulted in an adenine to cytosine nucleotide substitution and in the CCA codon for proline instead of the ACA codon for threonine. The complete nucleic acid and amino acid sequences are shown in Figure 14.

Transfection of Spodoptera frugiperda (Sf9) cells with pAC373 and the *Autographa california* nuclear polyhedrosis virus (AcMNPV) DNA successfully produced recombinant virus. Supernatant containing the recombinant baculovirus was plaque assayed, plaque purified, and expanded to obtain high titered stocks. Plaque assay results show that stocks contained titers of $1.6x10^8$ pfu/ml of recombinant baculovirus. These high titers stocks were used to infect High Five insect cells at a multiplicity of infection (MOI) of about 5. Western blots (Sambrook *et al.* 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press. Plainview, NY) showed that High Five cells produce BVp42-K protein beginning at 18 hours post infection (data not shown). At 42 hours the protein concentrations reached their peak levels of about 3-4 mg/L and the insect supernatants were harvested. Purification by affinity chromatography using 5.2 Mabs or phenyl HIC followed by Ni-NTA removed extraneous proteins. Analysis of purified recombinant protein by silver stains and western blots showed that the recombinant protein could be purified from insect cell supernatants and was present in high quantities (Figures 15 and 16).

Results of the BCA Protein Assay (Pierce, Rockford, IL) showed that reasonable amounts of purified protein are attainable. Affinity chromatography with monoclonal 5.2 antibodies yielded a protein concentration of 670 μg/ml at the peak aliquots. Phenyl HIC chromatography followed by Ni-NTA chromatography yielded lower concentrations of protein (<100 μg/ml) at peak aliquots. The histidine tag does not seem to be binding efficiently to the Ni-NTA resin as can be seen by the amount of residual material in western blots of the column filtrate (Figure 17). While the reason for this is unknown, it can be speculated that the histidine tag is not fully exposed in BVp42-K. Interestingly, even the denaturation of the protein with 6 M urea did not increase the recovery of purified protein.

Example 3

Comparison of Polyclonal Antibodies

Aotus lemurinus griseimembra monkeys (n=5) and New Zealand white rabbits (n=5) were immunized with BVp42 protein from the original BVp42 construct (the MSP-1 p42 construct obtained from the FUP isolate of *P. falciparum* [representing the MAD 20 "M" allele]) as described by Chang *et al.* 1996. Their antibodies were tested for reactivity with recombinant BVp42 and BVp42-K using indirect ELISA. Absorbancies were plotted on a scatter graph against the reciprocal of the antibody dilutions. Data points were adjusted into a linear relationship by changing the reciprocal dilution axis into a logarithmic scale. Data points for which the reciprocal dilution was greater than $4x10^6$ were not used due to non-linearity at these dilutions. A regression line for the data series was constructed and the equation of the line calculated. The equation was solved for the X-intercept to determine the titer of serum samples. A test for difference scores was performed on the data to determine if any significant differences existed between antibody titers of BVp42 and BVp42-K (Koosis DJ. 1997. Statistics: A Self-Teaching Guide. John Wiley and Sons Inc., New York, New York).

Table 2

ELISA Titer Comparison of Monkeys and Rabbits Immunized with BVp42 for BVp42 and BVp42-K

Monkey	BVp42	BVp42-K	Ratio K/M	Difference
3005	1310000	2340000	1.79	-1030000
82055	1060000	1660000	1.57	-600000
81591	390000	360000	0.92	30000
3006	670000	480000	0.72	190000
414	520000	620000	1.19	-100000
Average	790000	1092000	1.38	-302000
Rabbit	BVp42	BVp42-K	Ratio K/M	Difference
K192	2600000	2380000	0.92	220000
K191	1590000	1860000	1.17	-270000
K171	1430000	1670000	1.17	-240000
K170	1210000	1380000	1.14	-170000
K151	1730000	2100000	1.21	-370000
Average	1712000	1878000	1.10	-166000

The results showed that *Aotus* monkey sera reacted well with both the homologous BVp42 and heterologous BVp42-K proteins (Table 2). Antibody titers of BVp42 ranged from $3.9x10^5$ with monkey 81591 to $1.31x10^6$ with monkey 3005. BVp42-K titers ranged from $3.6x10^5$ with monkey 81591 to $2.34x10^6$ with monkey 3005. The average titer of the five monkeys with BVp42 was $7.9x10^5$ with the average titer with BVp42-K was $1.09x10^6$. The "*t*" test difference score result showed that antibody titers with BVp42 were not significantly different from BVp42-K using a significance level of 5% (α=0.05). Monkeys were able to recognize both MAD20 and K1 types of BVp42 even though two-thirds of the protein differs in amino acid sequence. However, the K/M ratios between individual monkeys varied slightly indicating that there may be slight differences in the specificity of antibody populations produced by individual animals.

Rabbit sera reacted equally well with the heterologous BVp42-K and the homologous BVp42 (Table 2). Antibody titers of BVp42 ranged from $1.21x10^6$ with rabbit K170 to $2.60x10^6$ with rabbit K192. BVp42-K titers ranged from $1.38x10^6$ with rabbit K170 to $2.38x10^6$ with rabbit K192. The average titer of the five rabbits with BVp42 was $1.71x10^6$ while the average titer with BVp42-K was $1.88x10^6$. Like the monkey results, "*t*" test difference scores show that antibody titers with BVp42 were not significantly different than BVp42-K using a significance level of 5% (α=0.05), emphasizing the fact the p42 dimorphism may not play an important role in B cell antigen recognition and antibody specificity. The rabbit K/M ratios were not as varied as the monkey K/M ratios but were similar averaging 1.1 and 1.4 respectively.

Example 4

Comparison of Monoclonal Antibodies

A panel of mouse Mabs made against the p42 region of MSP-1 was used to examine potential conformational differences between BVp42 and BVp42-K. These antibodies have been previously characterized and the areas of BVp42 reactivity established (Kaslow *et al.* 1993. Mol. Biochem. Parasitol. 63:283-2389). Mabs 4.2, 94-115, AD9.1, BC9.1, 91-33 and 91-115 all recognize conformational epitopes found in the conserved 19 kDa portion of BVp42. G13 is the only Mab found outside of the 19 kDa region and recognizes a linear epitope. Results from western blots with the various Mabs show that all of the antibodies react with both BVp42 and BVp42-K except for G13 (Figures 18A and 18B). Western blots show that G13 does not react with BVp42-K even though it has a high affinity for BVp42. Further tests with G13 (data not shown) using an ELISA, indicate that G13 may react very slightly with BVp42-K but not near the extent it reacts with BVp42. This slight cross-reactivity was unexpected since G13's epitope is located in the variable region of the 33 kDa area of BVp42 and was thought to be non-reactive with BVp42-K. Perhaps the dimorphism that exists in the N-terminal portion of the protein makes this epitope only very slightly accessible to G13 or perhaps a similar but not identical epitope exists on BVp42-K.

The positive results obtained with the other Mabs show that, despite its sequence differences at the N-terminus BVp42-K C-terminus seems to have the same general structure as the 19 kDa region of MSP-1. The 19 kDa region corresponding to block 17, which is considered to be a conserved region of MSP-1, contains four amino acid sties that can vary between strains. Antibodies used here (except for G13) recognize the conserved, conformational epitopes of the 19 kDa region (Kaslow *et al. supra*). This is important since the disulfide bridges found in the 19 kDa region play an important part in the tertiary structure of the protein. More importantly, the polyclonal antibody data presented here and by Hui *et al.* 1993. Infect. Immun. 61:3403-3411) suggest that the vast majority of BVp42 antibodies are made against the conserved portions of the protein, thus possibly down-playing the role of dimorphism in antibody recognition.

Example 5

Glycosylation Analysis

Visualization of BVp42-K with silver stains and western blots revealed that two forms of the protein were being produced by insect cells. Contamination with BVp42 was easily ruled out because neither form of BVp42-K reacted strongly with Mab G13, while BVp42 reacts strongly with the antibody. Glycosylation tests revealed that both BVp42-M and

BVp42-K are glycosylated in the baculovirus expression system. Both silver stains and western blots reveal that digestion of the proteins with N-glycosidase F results in a shift of each of the proteins to a lower molecular weight (Figure 19A-B). More significantly, incubation of N-glycosidase F with BVp42-K resulted in the resolution of the two form of the protein into one form of a smaller molecular weight. The shift to a lower molecular weight was less dramatic but still significant for BVp42. This difference corresponds to the presence of two potential N-glycosylation sites in the BVp42-K p42 sequence and only one potential N-glycosylation site in the BVp42 construct. Interestingly, this molecular weight shift was not seen in parasite isolated MSP-1, confirming other reports that N-glycosylation may not occur in P. falciparum (Blackman *et al.* 1991. Mol. Biochem. Parasitol. 49:2934; Holder *et al.* 1992. Mem. Inst. Oswaldo Cruz 87:37-42).

Tunicamycin experiments also supported the presence of N-glycosylation for both proteins in insect cells. Incubation of High Five cells with tunicamycin during recombinant baculovirus infection with either construct resulted in the same molecular weight shift of the expressed protein as seen with the N-glycosidase F (Figure 20). Incubation of BVp42-K with other glycosidases (O-glycosidase, β-galactosidase and neuraminidase) did not result in a molecular weight shift of the protein visualized by silver staining.

Example 6

Production and Purification of Yeast p42 (Yp42)

A yeast p42 construct was expressed in the alcohol dehydrogenase 2/glyceraldehyde-3-phosphate dehydrogenase (ADH_2-GAPDH) regulated expression system of *Saccharomyces cerevisiae* and has been described fully in Hui *et al.* 1991. J. Immunol. 147:3935-3941, expressing incorporated by reference. The p42 construct was based on the gp195 coding region from Ala_{1333}, to Ser_{1705} of the FUP isolate (Chang *et al.* 1988. Exp. Parasitol. 67:1). The yeast p42 polypeptide (Yp42) was purified using an affinity chromatography technique described by Siddiqui *et al.* 1987 for the purification of parasite gp195, except that polyclonal rabbit anti-gp195 IgG was used for antigen purification instead of anti-gp195 Mab 5.2, because the Yp42 protein could not be recovered in significant amounts using this Mab.

Figure 2B shows silver stains (lane 1) and immunoblots (lane 2) of the affinity chromatography purified Yp42. The silver stained antigens were electrophoresed in SDS polyacrylamide gels. The immunoblots were reacted with a rabbit anti-parasite gp195 serum pool. In the silver stain, a major species corresponding to Yp42 migrated as a 44

kDa molecule, although other minor bands were also present in varying amounts. In the immunoblots, the purified Yp42 displayed major immunoreactive species at the positions of the major protein bands, accompanied by minor reactivities with proteins of higher and lower molecular weight.

Example 7

Isolation of Purified, Parasite gp195 with Selected Fragments that Induce Substantially Complete Protection Against *Plasmodium falciparum* Challenge

A mixture of gp195 protein and certain of its processing fragments were obtained from *in vitro* cultured parasites (*P. falciparum* Uganda Palo Alto strain) using monoclonal-antibody affinity chromatography procedures employing Mab 5.2 (Siddiqui *et al.* 1987); *i.e.* the mixture is enriched in the epitope for which Mab 5.2 is specific. Importantly, this mixture has been previously shown to be capable of inducing substantially complete protection against a homologous challenge of *Plasmodium falciparum* in *Aotus* monkeys (Siddiqui *et al.* 1987). References in the examples below to "gp195" refer to the Mab 5.2-purified mixture of gp195 enriched with certain of its processing fragments.

In summary, saponin-lysed parasites were extracted with 1% NP-40 and the lysate was clarified by ultracentrifugation. The extracts were passed through a Protein G Sepharose column covalently conjugated with gp195-specific Mab 5.2 (USPN 4,897,354: expressly incorporated by reference and deposited with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland, USA 20852, on July 17, 1986 under accession number HB 9148). Mab 5.2 is specific for an antigenic determinant contained in p42, as demonstrated below.

After extensive washing to remove non-specifically bound material, specifically-bound proteins were eluted with 0.1 M glycine (pH 2.5) and neutralized with 1 M Tris-HCl (pH 8.0). The purity of the isolated gp195 was examined by SDS-PAGE followed by silver staining.

Example 8

In vitro Inhibition Assay

Substantially complete parasite inhibition was found obtainable using BVp42 as an immunogen in *in vitro* parasite growth inhibition assays. This level of inhibition has only been observed by us to be induced by the Mab 5.2 affinity-purified mixture of gp195 and fragments (Hui *et al.* 1987. Exp. Parasitol. 64:519). *In vitro* parasite growth inhibition

assays were performed by culturing parasites in the presence of immune rabbit serum using established methods (Hui *et al.* 1987. Exp. Parasitol. 64:519). Briefly, parasites were cultured in the presence of 15% preimmune serum or immune rabbit serum obtained 14, 21, 28, and 35 days after the fourth immunization with BVp42 in Complete Freund's Adjuvant (indicated as 4D14, 4D21, 4D28 and 4D35 in Table 3). The starting parasitemia (S) in each of the experiments was 0.2%. Growth inhibition was calculated according to the following equation:

$$\% \text{ inhibition} = \frac{(P - S) - (T - S)}{(P - S)} \times 100\%, \text{ where}$$

P = % parasitemia of cultures containing 15% preimmune serum at 72 hours;
T = % parasitemia of cultures containing 15% immune serum at 72 hours; and
S = starting % parasitemia of cultures at 0 (zero) hours.

Quaternary sera were used in inhibition assays. The corresponding pre-immune serum of each animal was used as a control. Parasite cultures were synchronized by sorbitol lysis (Lambros *et al.* 1979. J. Parasitol. 65:418) to select for late trophozoite and schizont stages. Infected erythrocytes were adjusted to an initial parasitemia of approximately 0.2% and a hematocrit of 0.8% with fresh erythrocytes. Rabbit preimmune or immune serum was added to a final concentration of 15%, and 200 µl of the culture suspension were added-in duplicate wells to a 96-well microtiter plate. Cultures were incubated at 37°C for 72 hours, and the parasitemia was determined by microscopy. The experiment was repeated three times for each of the rabbits used (rabbits given identification nos. 131 and 132). Results are shown in Table 3.

Quaternary sera of rabbits immunized with Yp42 had no significant effect on *in vitro* parasite growth (data not shown). In contrast, significant inhibition was obtained with quaternary sera of several bleeding dates from both rabbits immunized with BVp42 (Table 3). Sera obtained from later bleedings of rabbit 132 (4D21, 4D28, 4D35) nearly completely inhibited parasite growth; similar levels of inhibition have been observed previously only with antisera against Mab 5.2 purified, parasite gp195 (Hui *et al.* 1987).

Table 3

In Vitro Parasite Growth Inhibition Assay with Rabbit Anti-BVp42 Sera

Rabbit Serum	% Parasitemia (% Inhibition)		
	Expt. 1	Expt. 2	Expt. 3
Anti-BVp42 (131):			
Preimmune	13.3	6.8	9.8
4D14	10.3 (23)	--	--
4D21	5.6 (58)	3.9 (44)	4.0 (60)
4D28	--	1.7 (77)	2.6 (75)
4D35	--	1.6 (79)	1.9 (82)
Anti-BVp42 (132):			
Preimmune	9.0 (11)	6.7	8.7
4D14	5.4 (47)	--	--
4D21	<0.1 (>99)	0.4 (98)	0.2 (100)
4D28	--	0.3 (98)	0.3 (99)
4D35	--	0.3 (98)	0.9 (92)

Example 9

Determination of Immunogenicities and Cross-reactivities of Recombinant Polypeptides

A. ELISA Titers of Rabbits Immunized with Purified Parasite gp195 Against gp195, BVp42 and Yeast p42.

Rabbits were immunized with BVp42, Yp42 or enriched gp195 mixture (*i.e.* enriched for gp195 and C-terminal containing processing fragments through Mab 5.2 affinity purification) to determine the immunogenicity of the recombinant polypeptides and the cross-reactivity of anti-recombinant p42 antibodies with native gp195.

Rabbits given identification nos. 103, 104, 106 and 115 were immunized with purified, parasite gp195 emulsified in Complete Freund's Adjuvant as described (Hui *et al.* 1987). Rabbits 131 and 132 were immunized intramuscularly four times at 21-day intervals with 50 μg of purified BVp42 in Complete Freund's Adjuvant. Rabbits 93 and 96 were immunized intramuscularly five times at 21-day intervals with 50 μg of purified Yp42 in Complete Freund's Adjuvant. The amount of mycobacteria was reduced to one half of the first dose for the second immunization and one fourth of the first dose for subsequent immunizations, with the volume being replaced with Incomplete Freund's Adjuvant. Rabbits were bled before immunization and weekly after each immunization.

Serum antibodies produced against enriched gp195 mixture, BVp42, or Yp42 were assayed by an enzyme-linked immunosorbent assay (Chang *et al.* 1989; Chang *et al.* 1988) using the following technique. Vinyl plates were coated with purified, parasite gp195 and fragments (0.08 μg/ml), recombinant BVp42 (0.08 μg/ml), or recombinant Yp42 (0.2 μg/ml) and washed and blocked with 1% bovine serum albumin in borate buffered saline (BBS: 167 mM borate/134 mM NaCl, pH 8.0). Rabbit and mouse sera were serially diluted in 1% BSA/BBS, and human sera were serially diluted in phosphate-buffered saline (PBS: 150 mM sodium phosphate, 500 mM NaCl, pH 7.4) containing 0.05% Tween 20, 1.5% powdered milk, 0.05% BSA and 0.05% thimerosal. Diluted sera were added to antigen-coated wells and incubated for 1 hr at room temperature. Plates were washed with BBS containing 0.5 M NaCl (HSBBS) (rabbit and mouse sera) or PBS with 0.05% Tween 20 (human sera), and an appropriate dilution of peroxidase-conjugated species-specific anti-IgG (heavy and light chain specific) was added and incubated for 1 hr (anti-rabbit and anti-mouse IgG) or 2 hrs (anti-human IgG) at room temperature. Plates were washed in HSBBS and finally in BBS. One hundred microliters of peroxidase substrate solution [H_20_2 and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonate)] were added to each well, and the absorbance value at 410 nm was determined with a Dynatech 605 ELISA reader. The endpoint of ELISA titers for rabbit sera was designated to be the serum dilution producing an absorbance value of 0.2, which corresponded to twice the background O.D. reading. The ELISA endpoint titers for human sera was designated to be the serum dilution producing an absorbance value greater than 0.056, which was two standard deviations above the average reading for normal human sera.

A comparison of the reactivity of the polyclonal antisera from rabbits immunized with the purified, enriched parasite gp195, BVp42, and Yp42 in an ELISA is shown in Table 4.

Table 4

ELISA Titers of Rabbits Immunized with Purified, Parasite gp195 Against Parasite gp195, Baculovirus p42, and Yeast p42

Rabbit Serum	gp195 Titer	BVp42 Titer	Yp42 Titer
103	1/140,000	1/5,400	1/1,000
104	1/300,000	1/7,000	1/1,300
106	1/330,000	1/20,000	1/3,000
115	1/160,000	1/4,200	1/800

The highest ELISA titers were obtained against parasite gp195, the immunogen, with lower titers against BVp42, and the lowest titers against Yp42. These results are consistent with the expectation that antibodies are induced by gp195 outside of the p42

fragment, leading to a higher titer against the gp195 preparation than against BVp42.

B. ELISA and IFA Titers of Rabbits Immunized with BVp42 and Yeast p42 Against Parasite gp195, BVp42 and Yp42.

Additional rabbits were immunized with purified BVp42 (nos. 131, 132) or purified Yp42 (nos. 93, 96) to determine the immunogenicity of the recombinant polypeptides and the cross reactivity of anti-recombinant p42 antibodies with native gp195. Rabbit sera were tested by ELISA, parasite indirect immunofluorescence (IFA), and immunoblotting with parasite gp195.

The IFA procedure used was as follows. Assays were performed on acetone-fixed thin blood smears of schizonts and merozoites as described (Siddiqui *et al.* 1986. Infect. Immun. 52:314). Endpoint IFA titers corresponded to the final serum dilution producing parasite immunofluorescence above background levels observed using preimmune rabbit sera.

Sera were obtained on the day indicated (day 14, 21, 28 or 35, indicated as 4D14, *etc.* in Table 5 below) after four immunizations of rabbits with 50 μg BVp42 or 50 μg Yp42. Serial dilutions of sera were titered for reactivity with plates coated with recombinant p42 (rp42) or parasite gp195. "rp42" corresponds to BVp42 for rabbit anti-BVp42 sera and Yp42 for rabbit anti-Yp42 sera. The method of determining the ELISA endpoint titer for rabbit sera was as discussed above. Endpoint IFA titers correspond to the final serum dilution producing parasite immunofluorescence above preimmune serum backgrounds.

BVp42 proved to be highly immunogenic as shown in Table 5, inducing antibody titers comparable to those of rabbits immunized with purified, parasite gp195 (Table 2).

As shown in Table 5, ELISA titers were similar in assays utilizing plates coated with either purified, parasite gp195 or BVp42. Very high ELISA titers were obtained late in the quaternary response (days 28 and 35). Yp42 was less immunogenic than BVp42, inducing lower antibody titers against the immunogen. In addition, the cross-reactivity of anti-Yp42 antibodies with parasite gp195 in the ELISA was much lower than the cross-reactivity of anti-BVp42 antibodies. Typical merozoite surface staining patterns were observed by IFA for the BVp42 antisera (data not shown), and IFA titers obtained after the fourth immunization (Table 5) reached levels exceeding those obtained by immunization with purified, parasite gp195 (data not shown). Yp42 induced much lower

IFA titers.

C. ELISA Inhibition Assay

In order to obtain an estimate of the degree of cross-reactivity of anti-parasite gp195 (enriched mixture) antibodies with BVp42 and Yp42, we performed an ELISA inhibition assay utilizing the various antigens as inhibitors. The ELISA inhibition assay measures the reduction in reactivity of anti-parasite gp195 sera with parasite gp195 antigen in the presence of various concentrations of soluble parasite gp195 or recombinant p42. The ELISA inhibition assay was performed by diluting rabbit antisera to a point on the descending portion of the ELISA titration curve. The diluted sera (rabbit anti-parasite gp195 sera 103, 104, 106, and 115) were mixed with various concentrations of inhibitor (gp195, BVp42, or Yp42), incubated for 1 hour, and added to purified, parasite gp195-coated plates. Subsequent steps in the ELISA inhibition assay were identical to the standard ELISA assay described in section A above.

Table 5

ELISA and IFA Titers of Rabbits Immunized with Baculovirus p42 and Yeast p42 Against Parasite gp195, Baculovirus p42 and Yeast p42

Rabbit Serum	rp42 ELISA Titer	Gp195 ELISA Titer	IFA Titer
Anti-BVp42 (131):			
4D21	1/100,000	1/30,000	1/25,600
4D28	1/120,000	1/130,000	1/51,200
4D35	1/98,000	1/160,000	1/204,800
Anti-BVp42 (132):			
4D21	1/74,000	1/85,000	1/51,200
4D28	1/120,000	1/300,000	1/102,400
4D35	1/88,000	1/300,000	1/204,800
Anti-Yp42 (93):			
4D14	1/60,000	1/2,000	1/1,600
4D21	1/40,000	1/2,000	1/3,200
4D28	1/50,000	1/3,000	1/6,400
Anti-Yp42 (96)			
4D14	1/25,000	1/900	1/800
4D21	1/23,000	1/800	1/3,200
4D28	1/30,000	1/700	1/6,400

The results are shown in Figure 4. Soluble parasite gp195 completely inhibited the binding of anti-gp195 sera in this assay. A high level of inhibition (82-92%) was also obtained with soluble BVp42. Much lower levels of inhibition were seen with soluble Yp42 (28-47%). The average antigen concentration required to obtain 50% inhibition in the ELISA was similar for parasite gp195 (0.03 μg/ml) and BVp42 (0.04 μg/ml), while >5 μg/ml Yp42 was required for 50% inhibition. These results suggest that a majority of antibodies against parasite gp195 also recognize BVp42, i.e. purified, parasite gp195 and BVp42 are highly cross-reactive.

D. Reactivity with Human Sera

The recognition of gp195 (without Mab 5.2 affinity purified fragments) and related recombinant polypeptides by serum antibodies of individuals from a malaria-endemic area of the Philippines have been analyzed by Kramer and Oberst. Several individuals from this study were examined for antibody reactivity with parasite gp195 (Mab 5.2 affinity purified), BVp42 and Yp42. The results are shown in Table 6

Table 6

Reactivity of Human Sera from a Malaria-Endemic Area with Purified, Parasite gp195, the Baculovirus p42 Polypeptide, and the Yeast p42 Polypeptide

Individual	gp195 ELISA Titer	BVp42 ELISA Titer	Yp42 ELISA Titer
14270	1/100	1/200	neg.
14103	1/102,400	1/51,200	1/400
14122	1/102,400	1/51,200	1/400
14184	1/3,200	1/3,200	1/800
13563	1/200	1/1,600	neg.
13691	1/51,200	1/12,800	1/200
14187	1/21,800	1/12,800	1/100

Serial serum dilutions were tested for reactivity with the plates coated with parasite gp195, BVp42 and Yp42. Endpoint titers were designated as the serum dilution producing an O.D.>0.056, which was two standard deviations above the average reading for normal human sera. There was an excellent correlation between ELISA titers obtained with parasite gp195 and BVp42 (Pearson's correlation coefficient $r=0.96$, $p<0.001$). A lower correlation was obtained for gp195 and Yp42 ELISA titers (Pearson's correlation coefficient $r=0.86$, $p<0.01$). Thus, BVp42 cross reacts with serum antibodies of humans

from the malaria endemic area of the Philippines.

E. Recognition of Conformational Determinants by Antibodies of Rabbits Immunized with Parasite gp195 and Recombinant p42 and by a gp195 specific, Mab 5.2.

Figure 3, Panels A and B show immunoblots of purified parasite gp195 electrophoresed under nonreducing (panel A) or reducing (panel B) conditions in a 11.5% SDS-polyacrylamide gel. Immunoblots were reacted with the following antibody preparations: lane 1, anti-gp195 Mab 5.2; lane 2, rabbit anti-parasite gp195; lane 3, rabbit anti-BVp42 (#131); lane 4, rabbit anti-BVp42 (#132); lane 5, rabbit anti-Yp42 (#93); lane 6, rabbit anti-Yp42 (#96). Panel C: Immunoblots of BVp42 (lane 1, non-reduced; lane 2, reduced) and Yp42 (lane 3, non-reduced; lane 4, reduced) reacted with Mab 5.2.

The procedure for immunoblotting was as follows. Purified gp195 and BVp42 polypeptides were dissolved in Laemmli's buffer with or without 2-mercaptoethanol as a reducing agent and separated on $NaDodSO_4$ polyacrylamide gels (Laemmli. 1970. Nature 227:680). The separated proteins were electrophoretically transferred to nitrocellulose (Towbin *et al.* 1979. Proc. Natl. Acad. Sci. USA 76:4350) and reacted with rabbit and mouse antisera as described by Chang *et al.*, 1989.

Figures 3A-B, lane 1, show that Mab 5.2 is specific for a conformational determinant of the p42 fragment of gp195 since reactivity with this antibody was diminished by reduction of the parasite antigen. Figure 3B lanes 2, 3, and 4 also demonstrates that reactivity with the p42 processing fragments of both anti-gp195 sera and anti-BVp42 sera was markedly decreased when parasite gp195 was electrophoresed under reducing conditions. This indicates that both these antisera primarily recognize disulfide, conformational determinants of parasite gp195. In contrast, reactivity of anti-Yp42 sera, as shown in Fig. 3B (lanes 5 and 6) was slightly enhanced for reduced gp195, and the reduced 19 kDa processing fragment was recognized by these sera. Thus, the anti-Yp42 appears to recognize gp195 epitopes that are not disulfide-dependent.

The strong reactivity of BVp42 with Mab 5.2, as shown in Figure 3C, lane 1, and the loss of this reactivity by reduction of BVp42, as shown in Figure 3C, lane 2, indicates that the conformation of BVp42 closely resembles that of the native protein.

Example 10

Recognition of BVp42 by Antibodies from Congenic Mouse Strains

It has previously been shown that responsiveness to purified, parasite gp195 is present in mice of diverse major histocompatibility complex makeup (Chang *et al.* 1989). We determined whether the BVp42 antigen would be similarly recognized by anti-gp195 antibodies from a panel of congenic mouse strains.

Sera of seven congenic mouse strains possessing different H-2 haplotypes on the B10 background and that had been immunized with purified, parasite gp195 were tested for reactivity with the BVp42 antigen (Figure 5). Congenic mice of the following strain designations differing in H-2 haplotype but sharing the C57BL/10 genetic background were immunized: C57BL/10 SnJ, Bl0.A/SgSnJ, Bl0.D2/nSnJ, Bl0.M/SN, Bl0.WB (69NS), Bl0.BR/SgSnJ, and Bl0.PL (Jackson Laboratories, Bar Harbor, ME). Five mice per group were immunized intraperitoneally four times at 2-week intervals with 5 μg of purified, parasite gp195 emulsified in Complete Freund's Adjuvant. Mice were bled before immunization, on days 7 and 10 after the first immunization and on days 5, 7, and 14 after subsequent immunizations.

All seven strains produced anti-gp195 antibodies recognizing epitopes of BVp42 with some variation in titer among strains. Thus, similar to parasite gp195, individuals of diverse MHC haplotypes are capable of producing antibodies recognizing BVp42.

Example 11

Reactivity of Anti-BVp42 with Homologous vs. Heterologous Antigens

Since the gp42 processing fragment contains both conserved and allelic determinants, the reactivity of anti-BVp42 antibodies with homologous vs. heterologous gp195 antigens was characterized. Four parasite isolates, FUP, FVO, Hond-1 and Pf857 were used and by Southern blot analyses with allele specific oligonucleotide probes showed that FUP and Pf857 belong to the MAD allele, and FVO and Hond-1 belong to the K1 allele. Native gp195 antigens were purified by affinity chromatography from FUP and FVO parasites. In ELISAs, identical titers and binding curves were obtained with anti-BVp42 antibodies using either FUP (homologous) or FVO (heterologous) gp195 as antigens. Similar results were obtained in indirect immunofluorescent assays with FVO and FUP merozoites. More importantly, anti-BVp42 antibodies strongly or completely inhibited the *in vitro* parasite growth of the heterologous parasites (FVO and Hond-l) to the same degree as with the homologous parasites (FUP and Pf857).

Example 12

Immunogenicity of Various Adjuvant Formulation of BVp42 in *Aotus* monkeys

Aotus lemurinus griseimembra monkeys of karyotypes II and III with no history of previous *P. falciparum* exposure were screened for lack of BVp42 antibody reactivity by enzyme-linked immunosorbent assay (ELISA), the ability of the sera to support *in vitro* growth of plasmodia, normal blood chemistry and hematology values, absence of blood and intestinal parasites, absence of cardiovascular abnormalities and overall good health. *Aotus* monkeys passing this screening process were stratified by gender before being randomly assigned to the control or experimental groups. All animals were maintained at the University of Hawaii Laboratory Animal Service facility in accordance with National Institutes of Health and institutional guidelines.

The recombinant BVp42 antigen is based on the C-terminal merozoite surface protein-1 (MSP1) sequence of the Uganda-Palo Alto *P. falciparum* isolated (FUP) (Chang *et al.* 1988) which is closely related to the MAD20 MSP1 allele. BVp42 corresponds to the p42 coding region from Ala_{1333} to Ser_{1705} cloned into the *Autographica californica* nuclear polyhedrosis virus polyhedron promoter-regulated expression system (Luckow *et al.* 1988). A similar construct, designated BVp42-K, was generated consisting of the corresponding p42 coding region of the Vietnam-Oak Knoll isolate which is closely related in sequence to the Wellcome-K1 MSP1 allele (T. Nishimura, unpublished data). The recombinant p42 polypeptides were isolated from culture supernates of baculovirus infected *Trichoplusia ni* cells by immuno-affinity chromatography as previously described (Siddiqui *et al.* 1987). BVp42 purity was assessed by silver staining of antigen preparations separated by polyacrylamide gel electrophoresis.

Native MSP-1 protein and its processing fragments were purified from *in vitro* cultured parasites by immuno-affinity chromatography (Siddiqui *et al.* 1987).

Immunizations consisted of 100 μg of BVp42 in 0.25-0.5 ml of bacteriostatic 0.9% saline solution (Abbott Laboratories, North Chicago, IL) formulated with the adjuvants: MF59 (Chiron Corporation), MTP-PE (CIBA-Geigy), Stimulon™ QS21 (Aquila Biopharmaceuticals), or Montanide™ ISA51 oil-in-water emulsion (Seppic Inc.) according to the manufacturers instructions. In the case of MF59, immunizations were administered on weeks 0, 3, 6 and 9. An extended immunization protocol was used for MTP-PE + MF59 (MTP-PE: mutamyl tripeptide covalently linked to dipalmitoyl phosphatidyl ethanolamine; MF-59: an oil-in-water emulsion containing squalene, polysorbate 80 (Tween-20), sorbitan trioleate (Span85), and thimersol) for which

immunizations were administered on weeks 0, 8, 24 and 33. Monkeys received 200 μg MTP-PE in 0.5 ml MF-59 for each immunization (Keitel *et al.* 1993. Vaccine 11:909-913). For both QS-21 and ISA51 formulations, immunizations were given on weeks 0, 4, 8, 17, and 21. Each dose was given intramuscularly into alternating left and right thigh areas. In the first experiment, approximately 3 ml blood samples were collected from the femoral vein 14 days after each immunization and, for experiment 2, blood samples were collected monthly between the secondary and tertiary immunizations. Unimmunized animals or animals immunized with adjuvant alone served as controls.

Table 7

Experimental Groups Receiving various Adjuvant Formulation of the BVp42 Merozoite Surface Protein 1 Vaccine

Adjuvant Group	Adjuvant	Animal Nos.
I	MF-59	80140, 80315
II	MTP-PE + MF-59	84017, 84033, 84034, 84038
III	QS21	3006, 414, 81591, 82055
IV	Montanide ISA-51	3005, 81194, 82115

All of the adjuvant formulations tested were generally well tolerated and produced no systemic side effects in vaccinated monkeys. Local effects produced from immunization with BVp42/MF-59 included persistent induration at the site of injection and enlarged inguinal lymph nodes. Animals immunized with BVp42/MTP-PE + MF59 also developed enlarged inguinal lymph nodes but no induration at the site of injection. No local effects were observed at the site of injection or in the area of the regional lymph nodes for the BVp42/QS21 and BVp42/ISA51 formulations.

Figures 8A-D present the kinetics of the antibody response to BVp42 induced by immunization with the various adjuvant formulations. Significant boosting of antibody titer was observed after the second immunization with all four adjuvant formulations. Boosting was more variable after subsequent immunizations. For BVp42 in MF59, the peak titer for one animal (80315) was obtained after the third immunization while the titer for another animal (80140) continued to increase after the fourth immunization. Similarly, animals immunized with BVp42 in the MTP-PE+MF59 formulation developed peak titers after the third or fourth immunization although the titer of one animal (84017) reached its maximum after two immunizations, declining somewhat with subsequent immunizations. All of the animals immunized with BVp42/QS21 or BVp42/ISA51 continued to develop increased antibody levels with repeated immunization and achieved peak antibody titers

after four immunizations.

The cell mediated immune response induced by immunization was assessed by either antigen specific T-cell proliferation assays (Figure 9) or by the measurement of cytokine producing cells in antigen-stimulated **and unstimulated** ***** T-cell cultures (Figures 10A-B). Significant antigen-specific T-cell proliferation was observed for peripheral blood mononuclear cell cultures of animals injected with the three formulations tested using this assay (Figure 9: Groups I, II). Since cytokine production may be more informative than T-cell proliferation in assessing the type of T-cell response induced by the various adjuvant formulations, we also evaluated intracellular cytokine production in subsequent vaccination studies. Peripheral blood of *Aotus* receiving adjuvant formulations II, III, and IV contained high number of interferon-gamma (IFNγ) producing cells (Figure 10A). Significant numbers of IL-4 and IL-10 producing cells were also detected in unstimulated peripheral blood CD4+ cells.

Effectiveness of T cell priming with the two adjuvant formulations, BVp42/ISA51 and BVp42/QS21, was evaluated by enumeration of intracellular cytokine producing cells of these animals that were unstimulated and BVp42 stimulated *in vitro* (Table 8). Prior to initiation of immunization, the level of cytokine-producing cells in all animals were < 1% for IL-4 and IL-10 and 2% for IFNγ. Following immunization, significantly enhanced levels of cells producing the Th1 cytokine IFNγ and the Th2 cytokines IL-4 and IL-10 were detected in all immunized animals.

For animals immunized with BVp42/ISA51, a high percentage of IFNγ-producing cells (11-21%) were detected in unstimulated lymphocyte cultures of primed animals, indicating that immunization with this formulation induced an increase in peripheral blood cells producing this Th1 cytokine. Production of IL-10 was enhanced in BVp42-stimulated cultures as compared to cultures incubated without antigen, indicating that antigen-specific, primed T cells included a significant Th2, IL-10-producing component that could be expanded by BVp42 stimulation. In the other BVp42-stimulated cultures, the percentage of cytokine producing cells either remained the same (IL-4) or was slightly reduced (IFNγ) as compared to unstimulated cultures containing no antigen.

In the case of Aotus immunized with BVp42/QS21, IFNγ was also the predominant cytokine for most animals in terms of percentage of cytokine producing cells in unstimulated cultures. However, both IL-4 and IL-10 producing cells were enhanced upon antigen stimulation in vitro, consistent with effective priming of the Th2 antigen-

specific population with this formulation.

Table 8

Cytokine Production in Aotus Vaccinated with BVp42 Adjuvant Formulations

	Unstimulated			BVp42-stimulated		
Sample	IFNγ	IL4	IL10	IFNγ	IL4	IL10
BVp42/ISA51						
3005	20.5[a]	2.5	3	6	5.5	9
81194	11	1.5	1.5	4.5	1.5	5.5
82115	12	1.6	1.4	6.2	1.8	13.5
BVp42/QS21						
414	2.5	1	2.5	2.3	2	5.1
3006	7	0.8	0.5	4.5	1.6	3
81591	5.5	0.6	0.4	3.5	1.5	2
82055	19.5	2.2	8	14.5	8	16.5

[a] numbers correspond to the %CD4+ cells

In summary, the cytokine data indicate that both BVp42 adjuvant formulations induced priming of Th1- and Th2-like lymphocyte populations in vaccinated *Aotus*. While Th1-like IFNγ-producing cells comprised a major population of circulating lymphocytes in these animals, induction of antigen-specific Th2 cells producing IL-4 and IL-10 were also effectively induced with these formulations.

Example 13

In vitro Inhibition by Sera from Aotus Vaccinated with BVp42 Adjuvant Formulations

Purified serum immunoglobulin of *Aotus* immunized with the various adjuvant formulations were evaluated for biological activity in an in vitro assay *of P. falciparum* growth inhibition (Chang et al., Inf. Immun. 64:253-261, 1996). The results of two separate experiments are presented in Table 9. Preimmune immunoglobulin of all *Aotus* tested had no effect on in vitro parasite growth. A high level of growth inhibition (94.3% and 92.3%) was observed for the animal displaying the highest level of protective immunity, Aotus 81194, which was immunized with BVp42 in ISA51. A lower level of inhibition (82115) was noted for a second animal immunized with BVp42/ISA51 (Aotus 82115) although this animal showed no in vivo evidence of protective immunity. The third animal immunized with the BVp42/ISA51 formulation (Aotus 3005) showed a low level of inhibitory activity in one of the two experiments. Aotus immunized with the BVp42/QS21 formulation did not appear to produce significant levels of inhibitory antibodies as only low levels of inhibition were noted and inhibition was not consistent in

duplicate experiments. These results suggest that the in vitro growth inhibition assay may be used as a marker to identify individuals with a high level of immunity, as reflected by high levels of growth inhibition of immunoglobulin from Aotus 81194, and this may reflect one mechanism of protective immunity. However, this mechanism does not appear to be itself sufficient nor essential for protective immunity since (1) one animal displaying a detectable (albeit lower than 81194) level of inhibition showed no evidence of protection against parasite infection, and (2) several animals showing partial protection against parasite infection (81591 and 3005) did not show significant or consistent inhibition of in vitro parasite growth.

Table 9

In vitro Inhibition by Sera from Aotus Vaccinated with BVp42 Adjuvant Formulations

Animal No. (Vaccine Formulation)		Expt #1*		Expt. #2*	
		Parasitemia (%)	% Inh.	Parasitemia (%)	% Inh.
Media Control		3		3.8	
417 (BVp42/CFA)		0.4		0.6	
414 (BVp42/QS21)	Preimmune	2.2	Neg.	2.8	Neg.
	Immune	2.6		3.1	
3005 (BVp42/ISA51)	Preimmune	3	Neg.	3	21.4
	Immune	3.6		2.4	
3006 (BVp42/QS21)	Preimmune	2.8	Neg.	3.5	57.6
	Immune	2.8		1.6	
81194 (BVp42/ISA51)	Preimmune	3.7	94.3	2.8	92.3
	Immune	0.4		0.4	
81591 (BVp42/QS21)	Preimmune	3.3	Neg.	3	Neg.
	Immune	3.2		3	
82055 (BVp42/QS21)	Preimmune	3.8	16.7	3.4	Neg.
	Immune	3.2		3.6	
82115 (BVp42/ISA51)	Preimmune	3.3	29	3.8	41.7
	Immune	2.4		2.3	

Starting Parasitemia: 0.2%

Example 14

Aotus monkeys Immunized with BVp42 Adjuvant Formulations are Protected from a Lethal Challenge of *Plasmodium falciparum*

To generate the parasite challenge inoculum, a frozen stabilite of *in vivo*-passaged FUP isolate *P. falciparum* was thawed and inoculated into a malaria-naive *Aotus* monkey (Expts. 1 and 3) or into an *in vitro* culture of *Aotus* erythrocytes (Expt. 2). Peripheral blood containing parasitized erythrocytes was collected from infected animals or from overnight culture and adjusted to 7 x 10^5 parasites per ml, and 0.5 ml was injected into the saphenous vein of control animals and experimental animals at 1 week (Expt. 1), 3 weeks (Expt. 2) or 4 weeks (Expt. 3) after the final immunization. Parasitemias were monitored by Geimsa stained blood smears of peripheral blood. The experimental endpoint for these vaccination studies was defined as either parasitemia $\geq$10% erythrocytes, and erythrocyte count of $<3.0 \times 10^6$/ml, or declining health as determined by the attending veterinarian. Antimalarial therapy consisted of oral choloroquine (10 mg/kg of body weight per day for 4 days) or mefloquine (25 mg/kg of body weight, at one time). The experiment was terminated by antimalarial drug treatment of remaining, untreated animals 6 weeks after parasite challenge.

Prechallenge antibody titer values measured after the final immunization for the different adjuvant groups are presented in Table 10. High ELISA titers (>500,000) were achieved in all adjuvant groups. The highest ELISA titer (>10,000,000) was seen for an animal (81194) immunized with BVp42 in ISA51. At least one animal in each adjuvant group developed a prechallenge titer $\geq$1,000,000. Consistently higher antibody titers were noted for animals immunized with the MTP-PE+MF59 and ISA51 formulations than other adjuvant formulations. IFA titers followed a similar trend to the ELISA titers, ranging from 1:1600 in animals with the lowest ELISA titers to 1:6400 for the animal with the highest ELISA titer.

Table 10

Relationship of prechallenge anti-MSP1 antibody titers to characteristics of *P. falciparum* infection of *Aotus* immunized with BVp42 in various adjuvant formulations.

Experiment No.	Animal No.	Immunogen	Prechallenge Antibody Titers ELISA	IFA	Prepatent Period (days)	Pretreatment Period (days)
1	81570	-	-	-	3	8
	80469		-	-	4	12
	80140	BVp42/MF59	1,600,000	1,600	3	10
	80351		700,000	1,600	5	10
2	82016	-	-	-	3	11
	84028				7	13
	84029				7	13
	84017	BVp42/MTP-PE + MF59	850,000	800	7	13
	84033		4,000,000	3,200	7	13
	84034		3,000,000	3,200	7	13
	84038		2,000,000	3,200	7	13
3	416	-	-	-	5	7
	419				5	7
	81262				4	7
	414	BVp42/QS21	700,000	3,200	7	14
	3006		700,000	1,600	5	14
	81591		550,000	3,200	4	-*
	82055		1,000,000	3,200	5	8
	3005	BVp42/ISA51	2,900,000	3,200	8	20
	81194		>10,000,000	6,400	23	-*
	82115		3,100,000	3,200	5	8

*No treatment administered during experiment; animals self-cured.

Experimental animals immunized with the various adjuvant formulations along with normal controls (expt. 1 & 3) or adjuvant controls (expt. 2) were administered a lethal, intravenous dose of *Plasmodium falciparum* (Uganda-Palo Alto isolate) after the final immunization. The course of *P. falciparum* for these animals is presented in Figures 11A-D.

No significant differences in the course and final outcome of infection were seen between control and experimental *Aotus* immunized with the BVp42/MF59 (Fig. 11A) or the BVp42/MTP-PE + MF59 (Figure 11C) formulations. The time intervals between parasite injection and detection of peripheral blood parasitemia (prepatent period) and between challenge and drug-treatment (pretreatment period) were indistinguishable for control and experimental animals receiving these two formulations (Table 10).

In contrast, the course of *P. falciparum* infection was distinctly different for *Aotus* immunized with both BVp42/QS21 and BVp42/ISA51 formulations. For *Aotus* immunized with BVp42/QS21 (Fig. 11B), the prepatent period was similar to the normal controls but the course of infection and, in one instance, the outcome of infection were quite different. While parasitemias of control animals rapidly rose to treatment levels ($\geq$10%) within seven days after parasite challenge, the course of infection in three of the four animals in the experimental group was much slower than the control group. These animals experienced a prolonged period of controlled parasite multiplication, resulting in a significantly extended pretreatment period. For one animal in this group (81591), this phase of controlled parasitemia was followed by the gradual clearance of parasites from peripheral blood and self-cure of the infection.

The most striking results and strongest protection were obtained with *Aotus* immunized with BVp42/ISA51 (Fig. 11D). The course of infection of two of the three animals in this group (3005 and 81194) was markedly different from the controls. *Aotus* 3005 experienced a lengthened prepatent period as compared to that of the control animals, after which time its parasitemia increased to moderate levels but was controlled for an extended period of time, similar to the QS21 group. While the trend of declining parasitemia suggested that this animal was in the process of clearing the parasite infection, it was drug-treated on day 20 because of its impaired health status. The animal displaying the highest level of protective immunity was *Aotus* 81194 which had no detectable parasitemia until day 23 after challenge. Thereafter, its parasitemia slowly increased, reaching a maximum of 1% on days 9-10 post-challenge. Following this peak in parasitemia, the parasite numbers abruptly dropped to low levels ($<$ 0.05%) for a week before disappearing from peripheral circulation. Most notably, *Aotus* 81194

remained healthy and vigorous throughout the infection period, experiencing no reduction in its erythrocyte count (data not shown).

Table 11 presents the ELISA titers of animals immunized with BVp42/QS21 and BVp42/ISA and challenged with *P. falciparum* with three different solid-phase antigens: recombinant FUP BVp42 (BVp42-M) representing the homologous MAD20 MSP1 allele, recombinant FVO BVp42 (BVp42-K) representing the heterologous K1 MSP1 allele, and parasite-derived FUP MSP1. In the case of *Aotus* immunized with BVp42/QS21, similar titers were obtained using the homologous and heterologous BVp42 antigens and there was a high level of cross reactivity of antibodies produced against the recombinant p42 MSP1 and the homologous MSP1 purified from parasite extracts. The animal showing the highest level of immunity within this group (81591), as reflected by self-cure of the parasite infection, did not show a significant difference in specificity from others within the group. It is also interesting to note that the only animal showing no evidence of protective immunity to parasite infection in this group (82055), displayed a high ELISA titer for all three antigens.

Aotus 81194 immunized with BVp42/ISA51 developed the highest titers against all three antigens and displayed the highest level of protective immunity. While ELISA titers were similar for another animal in the group displaying protective immunity (3005) and an unprotected animal (82115), there appeared to be a difference in specificity between these two animals. Although the reactivity of *Aotus* 3005 was similar for the homologous and heterologous BVp42 antigens (BVp42-M:BVp2-K ratio = 1.3), the reactivity of *Aotus* 82115 was much lower for the heterologous than the homologous BVp42 antigen (BVp42-M:BVp42-K ratio = 2.1). These results suggest that while the majority of antibodies of *Aotus* 3005 (and *Aotus* 81194) may have been directed against conserved p42 epitopes, antibodies produced by *Aotus* 82115 may not be focused to the same extent on these protective, conserved epitopes.

The results of the present study show for the first time that a MSP p42 malaria antigen formulated with the adjuvants the Stimulon™ QS21 and Montanide™ ISA51 adjuvants are capable of inducing a protective immune response to *P. falciparum* infection.

Table 11

Comparison of ELISA titers of *Aotus* immunized with BVp42/QS21 and BVp42/ISA51 for homologous, recombinant BVp42-M, heterologous, recombinant BVp42-K, and homologous, parasite-purified MSP1.

Animal No.	Immunogen	ELISA Titer			
		MSP1	BVp42[a]	BVp42-K[b]	M:K Ratio
414	BVp42/QS21	1,600,000	700,000	600,000	1.2
3006		700,000	700,000	460,000	1.5
81591		900,000	550,000	360,000	1.5
82055		1,000,000	1,000,000	1,700,000	0.6
3005	BVp42/ISA51	1,800,000	2,900,000	2,300,000	1.3
82115		1,700,000	3,100,000	1,5000,000	2.1
81194		>10,000,000	>10,000,000	>10,000,000	~1

[a] BVp42 represents MSP-1 "M" allele

[b] BVp42-K represents MSP-1 "K" allele